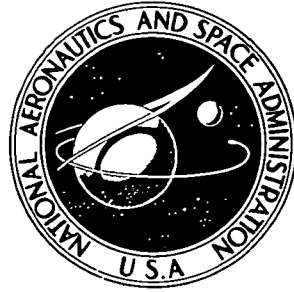


NASA TECHNICAL NOTE



NASA TN D-8439

NASA TN D-8439

**APPLICATION OF FIREFLY LUCIFERASE ASSAY
FOR ADENOSINE TRIPHOSPHATE (ATP) TO
ANTIMICROBIAL DRUG SENSITIVITY TESTING**

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1. Report No. TND-8439		2. Government Accession No.		3. Recipient's Catalog No.	
4. Title and Subtitle Application of Firefly Luciferase Assay for Adenosine Triphosphate (ATP) to Antimicrobial Drug Sensitivity Testing			5. Report Date July 1977		
			6. Performing Organization Code 726		
7. Author(s) G. L. Picciolo and E. W. Chappelle			8. Performing Organization Report No. G-7703		
9. Performing Organization Name and Address Goddard Space Flight Center Greenbelt, Maryland 20771			10. Work Unit No. 644-02-01		
			11. Contract or Grant No.		
12. Sponsoring Agency Name and Address National Aeronautics and Space Administration Washington, D.C. 20546			13. Type of Report and Period Covered Technical Note		
			14. Sponsoring Agency Code		
15. Supplementary Notes					
16. Abstract This report documents the development of a rapid method for determining microbial susceptibilities to antibiotics using the firefly luciferase assay for adenosine triphosphate (ATP). The reduction of bacterial ATP by an antimicrobial agent was determined to be a valid measure of drug effect in most cases. A basic procedure for determining microbial sensitivity evolved in which broth cultures of log phase bacteria (10^6 per ml) were grown for 2.5 hours at antimicrobial concentrations which resulted in the best discrimination between "sensitive" and "resistant" strains. Drug effect was quantitated by comparing ATP levels before and after incubation in the presence of antibiotic with ATP levels of an antibiotic-free growth control. The effect of 12 antibiotics on 8 different bacterial species gave a 94% correlation with the standard Kirby-Bauer agar disc diffusion method. A 93% correlation was obtained when the ATP assay method was applied directly to 50 urine specimens from patients with urinary tract infections. Urine samples were centrifuged first so that bacterial pellets could be suspended in broth. No primary isolation or subculturing was required. Mixed cultures in which one species was predominant gave accurate results for the most abundant organism. Since the method is based on an increase in bacterial ATP with time, the presence of leukocytes did not interfere with the interpretation of results. Both the incubation procedure and the ATP assays are compatible with automation.					
17. Key Words (Selected by Author(s)) Adenosine triphosphate, firefly luciferase ATP assay, antimicrobial susceptibility testing, antibiotics, urinary tract infections, bacteria			18. Distribution Statement Unclassified—Unlimited		
19. Security Classif. (of this report) Unclassified	20. Security Classif. (of this page) Unclassified		21. No. of Pages 160	22. Price* \$6.75	

All measurement values are expressed in the International System of Units (SI) in accordance with NASA Policy Directive 2220.4, paragraph 4.

PREFACE

The intention of this report is to document the development of a rapid method for determining microbial susceptibilities to antibiotics by use of the firefly luciferase ATP assay. The progress and results of the studies involved, including developmental decisions and raw data, are recorded chronologically in report form from the inception of the project through completion of efforts in 1975. The studies were conducted at the Tufts-New England Medical Center Hospital (NEMCH) in Boston under a cooperative agreement with Goddard Space Flight Center (GSFC), National Aeronautics and Space Administration (NASA). Laboratory and clinical results from this research have been summarized and presented at annual meetings of the American Society for Microbiology (Vellend et al., 1974; Schrock et al., 1975; Schrock et al., 1976) and published in the form of a NASA X-document (Picciolo et al., 1976). Appropriate patent applications have been filed in the U.S. Patent Office (Chappelle et al., Schrock et al., Picciolo et al.).

Further modifications, refinements, and clinical testing of procedures for detection of bacteriuria as well as determinations of antibiotic susceptibilities have continued at the Hahnemann Medical College and Hospital (HMCH) in Philadelphia, under a contract with NASA/GSFC. The major thrust of ongoing research centers on the development of filtration alternatives to centrifugation steps, reducing time and manipulations required in procedures as developed at NEMCH. HMCH results have been presented at various scientific meetings and conferences (Gutekunst, 1975; Gutekunst, 1977, Gutekunst, Jaffee et al., 1976; McGarry and Chappelle, 1976; Jaffee et al., 1976; Gutekunst, Picciolo et al., 1976; Deming et al., 1977; Gutekunst et al., 1977) and will be summarized in a future NASA X-document.

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APPLICATION OF FIREFLY LUCIFERASE ASSAY FOR ADENOSINE TRIPHOSPHATE (ATP) TO ANTIMICROBIAL DRUG SENSITIVITY TESTING

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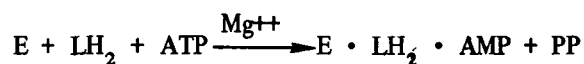
INTRODUCTION

The objective of the research to be described was to develop and evaluate a rapid method for the determination of microbial susceptibility to antibiotics using the firefly luciferase assay for adenosine triphosphate (ATP) as an indicator of antibiotic effect. The research was a direct outgrowth of an effort by National Aeronautics Space Administration to develop methods for detecting extraterrestrial life. In the 1960's, investigations undertaken by the Space Biology Branch of Goddard Space Flight Center (GSFC) had the following objectives:

- The determination of a parameter whose presence or absence would be a relatively universal indicator of life forms
- The development of techniques for the detection of this parameter in the presence of interfering substances
- The automation of these techniques so that the assay could be performed with high reliability in remote locations

The parameter chosen as a relatively universal indicator of life forms was the presence of ATP. The ubiquity and functional significance of ATP in metabolism allows its assay to be an excellent monitor of the biological mass of a specimen. Because the firefly luciferase bioluminescence reaction is specific for ATP, many investigators have used this reaction to determine the amount of bacteria or biological mass in a specimen. (Beutler and Mathau, 1967; Brewer and Knutsen, 1966; Cole et al., 1967; Ebadi et al., 1971; Freese et al., 1969; Holm-Hansen and Booth, 1966; Lee et al., 1971; Patterson et al., 1970).

The reaction mechanism and kinetics have been determined by several investigators (McElroy et al., 1969; Strehler and McElroy, 1968). The reaction can be summarized in two steps (Plant et al., 1968):



where	E	= firefly luciferase
	LH ₂	= reduced luciferin
	ATP	= adenosine triphosphate
	E · LH ₂ · AMP	= luciferase-luciferyl-adenylate complex (active intermediate)
	Oxyluciferin	= luciferin (oxidized state)
	PP	= pyrophosphate
	AMP	= adenosine-5' monophosphate
	hν	= light (550 nm)

Conditions have been prescribed where this enzymatic reaction can be used as a rapid, sensitive, and simple assay for the quantitation of bacteria (Chappelle and Levin, 1964). Several methods for the extraction of ATP from bacterial cells were developed (Chappelle and Levin, 1968; Klofat et al., 1969; Macleod et al., 1969). When all reagents are present in excess, the light production is proportional to the ATP concentration, which is in turn proportional to the bacterial cell concentration in the specimen. The plot of bacterial cell concentration versus light units shows a linear response over a functional range (Chappelle and Levin, 1968).

The average ATP content of a wide variety of bacterial species is 2.5×10^{-10} μg per organism. The ATP content varies somewhat through a growth cycle (Freese et al., 1969; Klofat et al., 1969) and varies with species from 0.28 to 8.9×10^{-10} μg per organism for the 19 species tested (Chappelle and Levin, 1968; Seliger, 1973).

One of the major problems in determining the relationship between ATP levels and bacterial cell numbers is the presence of significant amounts of soluble ATP and cellular ATP of non-bacterial origin in the sample under consideration. To isolate bacterial cells for assay, a method was developed to chemically remove the ATP associated with nonbacterial cells as well as any soluble ATP from a specimen (Picciolo et al., 1971). The specimen is treated with a nonionic detergent that selectively lyses mammalian cells but does not disturb the cell membranes of bacteria. An ATPase that hydrolyzes all the free ATP is added. The reaction is then inhibited, the bacteria are lysed, and their ATP content assayed by the luciferase bioluminescent reaction.

Potential biomedical applications of the ATP assay technology have been investigated by the Space Biology Branch and more recently the Instrument Branch of GSFC. In 1969, studies were undertaken to evaluate the ATP assay for the rapid detection of bacteria in urine and a prototype automated instrument was developed (Picciolo et al., 1971). Initially, over 700 urine specimens were assayed for bacteria by using both the ATP method and standard colony counting on agar plates. While the ATP method always gave positive results when the urine specimen contained greater than 10,000 bacteria/ml (a number generally considered to be clinically significant (Kass, 1956)), this method often gave values which corresponded to much higher bacterial counts than those obtained by plate counting. However, the quantitative correlation was significantly improved with refinements in the methods of eliminating nonbacterial ATP and by increasing the operational sensitivity of the reaction by concentration of the bacteria from the urine specimen. Randomly selected results from recent studies are shown in table 1. Investigations as to the sources of the discrepancies still seen between the ATP method and viable colony counting are currently in progress.

Table 1
Bacterial Count per ml from Clinical Urine Specimens
Comparing the Luciferase Centrifugation Procedure
with the Agar Pour Plate Method

Specimen Number	Luciferase Bacteria per ml	Pour Plate Colony Count
1	3.2×10^8	$>10^7$
2	3.1×10^4	4×10^3
3	$<10^3$	$<10^2$
4	$<10^3$	8×10^3
5	7.0×10^3	1×10^5
6	$<10^3$	$<10^2$
7	9.4×10^3	2×10^2
8	$<10^3$	$<10^2$
9	3.1×10^7	$>10^7$
10	3.1×10^7	$>10^7$
11	$<10^3$	$<10^2$
12	2.7×10^4	$<10^2$
13	8.4×10^7	$>10^7$
14	3.2×10^4	9×10^2
15	2.1×10^8	8×10^6
16	1.3×10^7	1×10^7
17	1.6×10^7	$>10^7$
18	8.6×10^4	4×10^4
19	$<10^3$	1×10^2
20	2.4×10^4	$<10^2$
21	3.1×10^6	5×10^6
22	5.5×10^3	1×10^3

Following a review of this work by the National Academy of Engineering's Committee on the Interplay of Engineering with Biology and Medicine, Dr. David Rutstein of the Harvard School of Public Health recommended that this technology be applied to the rapid determination of microbial susceptibility to antibiotics. A collaborative proposal for this purpose was drawn up by GSFC and the New England Medical Center Hospital, Boston Massachusetts. The progress and results of these studies are the substance of this document. A final summary of initial studies using pure bacterial cultures as opposed to clinical samples is included as Appendix A.

Optimum use of antimicrobial agents to combat infection depends upon knowledge of the susceptibility of the infecting organism. Although susceptibility can sometimes be predicted from the general behavior of the bacterial species involved, the most reliable approach is generally that of direct measurement of the *in vitro* interaction between the organism and the drug.

Present techniques of microbial sensitivity testing generally require overnight incubation after primary isolation of the infecting organism. This requires a minimum of 48 hours after the specimen is received in the laboratory. The methods most widely used are agar diffusion (Kirby-Bauer), broth dilution, and agar dilution (Ericsson and Sherris, 1971; Bauer et al., 1966; Anderson, 1970; Balows, 1974). Only the agar diffusion technique is reasonably well standardized and is widely used in clinical microbiology laboratories.

It is clear that the availability of a rapid method of testing bacterial susceptibility would permit prompt selection of the most effective agent and therefore avoid the inclusion of inappropriate, unnecessary—and often toxic—agents in the initial therapeutic regimen.

Although several rapid methods have been described in the past few years (Sellers, et al., 1969; Isenberg et al., 1971; McKie et al., 1973; Thornsberry et al., 1973; McGowan et al., 1973; Binford et al., 1973; DeBlanc et al., 1972; Deland, 1972; Boyle et al., 1973; Barry et al., 1973), all have shortcomings. The measurement of ATP lends itself as an index of bacterial growth because it can be used for screening specimens for a subsequent susceptibility testing; it can be made specific for bacterial ATP; with careful standardization of reagents, it is precise, reproducible, and compatible with automation; and an individual specimen can be assayed for its ATP content in less than 30 minutes.

Conventional susceptibility testing involving overnight incubation reflects the final result of a dynamic and complex interaction between the antimicrobial agent and the microorganism. All currently described methods attempting to rapidly quantitate drug effect will have a variety of problems in common, which arise from the particular characteristics of the bacterium measured, the mode of action of the antimicrobial agent, and the mechanism of resistance to the antimicrobial agent.

These phenomena are discussed in this document. As Dr. John C. Sherris has cautioned, "One problem will be to relate (the results of rapid, automated procedures) to those of more orthodox mean inhibitory concentrations (MIC) methods because of the varying early effects of sub-MIC concentrations of different chemotherapeutics on different organisms. This poses

the questions of whether such methods can be programmed to give comparable results to those of an overnight MIC and, perhaps more importantly, of the clinical significance of early microbial responses to chemotherapeutics as compared with overnight results. It is certainly possible that the results of an automated procedure may become the future standard against which other methods are judged" (Balows, 1974).

SECTION 1
PRELIMINARY PROTOCOL, MARCH 1973:
APPLICATION OF LUCIFERASE ASSAY
FOR ATP TO ANTIMICROBIAL DRUG
SENSITIVITY TESTING

INTRODUCTION

The purpose of the preliminary series of experiments is to establish that the inhibition (reduction) of bacterial ATP by antimicrobial agents is a valid measurement of drug effect. The initial protocol will be divided into four parts.

Part 1: Determination of Mean Inhibitory Concentrations (MIC)

At present, the MIC is the standard technique by which drug effect versus a specific microorganism is quantitated. Therefore, it is necessary to determine the MIC of several antimicrobial agents (penicillin G, ampicillin, tetracycline, sulfisoxazole, gentamicin, and cephalothin) widely used to treat urinary tract infections on representative bacterial species commonly involved in urinary tract infections. The representative bacterial species are *Escherichia Coli*, *Klebsiella*, *Enterobacter*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Streptococcus faecalis*.

Part 2: Effects of Antibiotics on the Amount of ATP per Viable Cell

The effect of the MIC and $2 \log_2$ above and below this concentration of the particular antibiotic on the log phase growth rate (as measured by the viable colony count) and the bacterial ATP concentration are measured. This is expected to yield some fixed measurable percentage end-point which will correlate well with the MIC. A standard initial concentration of $\sim 10^6$ organisms/ml will be used.

The purpose of starting with an evaluation of the MIC's for the organisms of interest is to be able to compare results from the ATP assay with those from the technique that is used as a standard method for evaluating susceptibilities, providing a data base for further work with urine specimens. It will evaluate the relationship between the viable plate count and determination of ATP content per bacterium growing in the presence of antibiotics. This will indicate if it is necessary to measure total count as a function of ATP and viable count to determine if there is a change in ATP per bacterium caused by the antibiotics.

Part 3: Effect of Inoculum Size on Antibiotic Activity

Since there is considerable range of bacterial concentration in infected urine specimens, the effect of varying initial bacterial concentrations (10^4 , 10^6 , and 10^8 /ml) is studied using the MIC concentration of the drug.

Part 4: Effect of Urine on Bacterial Growth Rates, ATP, and Drug Activity

Since the objective is to be able to apply the technique directly to infected urine specimens inoculated into broth, the effect of urine per se on the procedures evaluated in Parts 2 and 3 is studied. Pooled, filtered urine is used for these studies.

PROCEDURES

Part 1: MIC Determinations

1. The representative bacterial species (preferably American Type Culture Collection (ATCC) organisms) selected are grown in trypticase soy broth (TSB) at 37°C overnight.
2. Serial dilutions of the appropriate antibiotic are made in TSB (256, 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, and 0.125 µg/ml).
3. The initial concentration of the organism is $\sim 10^7$ (add 0.02 ml of overnight broth culture to 2 ml of broth containing antibiotic).
4. The tubes are incubated at 37°C for 18 hours, and the least concentration of antibiotic inhibiting visible growth of the organism will be the MIC.
5. Control organism of known MIC is run simultaneously.

Part 2: Growth Curves

1. An overnight broth culture of the test organism is diluted in 37°C TSB to give a concentration of $\sim 10^6$ organism/ml.
2. This is preincubated in a shaking water bath for 60 to 90 minutes to reach log phase.
3. To 9-ml aliquots of this broth culture is added 1.0 ml of the concentrated antibiotic solution to yield the required final concentration of antibiotic. The concentration evaluated will be the MIC concentration and 2 log₂ above and below this concentration. An antibiotic-free control is run simultaneously.
4. Viable colony counts are determined at 0, 2, 4, 6, and 24 hours using the replicate plate Miles-Misra counting method.
5. The noncentrifuged no-malic acid procedure (Chappelle et al., 1975) for bacterial ATP will be run on all the aliquots: organisms in broth, with and without the various antibiotics at different concentrations, at the various time periods, including 0 time, a broth blank with each antibiotic, and a broth blank control and a standard ATP in each broth, with and without antibiotics.

As experience is gained in working with the blanks and antibiotics, some of them may be eliminated.

6. GSFC has optimized a procedure for use in these studies to determine the ATP levels in the growing bacteria (Chappelle et al., 1975). This will involve use of a nonconcentrating procedure using Triton X-100 (TX-100), apyrase, calcium, and the optimal concentration of HNO_3 . The use of malic acid buffer to remove residual ATP would introduce the complication of reducing the intrabacterial ATP. Since in pure species work, the amount of extrabacterial bound ATP is expected to be small, there should be no need to use the malic acid. The noncentrifuged procedure without malic-arsenate for luciferase assay of bacteria follows:
 - a. Prepare a 0.5-ml sample: broth culture of bacteria, etc.
 - b. Add 0.1 ml of Apy-Ca-Tx (10 mg Apyrase/ml 0.03 M CaCl_2 -0.6 percent TX-100); wait 15 minutes; vortex well.
 - c. Add 0.1 ml 1.5 N HNO_3 ; wait 5 minutes; vortex well.
 - d. Add 4.3 ml H_2O ; mix well.
 - e. Assay: Inject 0.1 ml of the above sample into 0.1 ml of DuPont luciferase reconstituted with 1.5 ml of 0.1 M TRIS (hydroxymethyl) aminomethane, 0.01 M MgSO_4 , pH 8.0.
 - Recovery: Add 0.05 ml of 10 or 1 mg ATP/ml, depending on desired accuracy of recovery, to the sample after the initial assay, and again inject for an assay of this standard.

Part 3: Inoculum Size

The previous experiment is repeated using a control and three different initial bacterial concentrations at a fixed concentration of drug (MIC). The purpose of this is to evaluate the differences in inhibition of ATP concentration at varying inoculum sizes which are representative of the range found in infected urines (10^4 , 10^6 , and 10^8 organisms/ml).

Part 4: Effect of Urine

1. Pure cultures of organisms in pooled, filtered urine will be prepared at the various expected concentrations (10^4 , 10^6 , and 10^8 organisms/ml).
2. These cultures will be added to broth in various ratios to determine optimal conditions for rapid bacterial multiplication.
3. The effect of the presence of urine at the optimal growth ratios on the ATP assay will be determined.

SECTION 2
INTERIM PROGRESS REPORT, JULY 1973:
INITIAL PHASES OF THE APPLICATION—MEAN
INHIBITORY CONCENTRATIONS (Mic)

INTRODUCTION

The initial phases of the preliminary protocol were completed in July 1973. The effect of the MIC, 0.25 MIC, and four times the MIC of gentamicin and tetracycline were measured on the eight microbial species selected for evaluation.

Summary of Results

There is a readily-measured, dose-related effect of gentamicin and tetracycline on both the viable colony counts and bacterial adenosine triphosphate (ATP) in the first 2 to 3 hours of log phase growth (figures 2-1 through 2-4 and tables 2-1 through 2-4). The mean doubling time of the control culture was 30 minutes. The antibiotics evaluated interfere with the ability of the bacterium to maintain its intracellular ATP concentration. The magnitude of the drug effect on ATP is less pronounced than its effect on the ability of the organism to multiply.

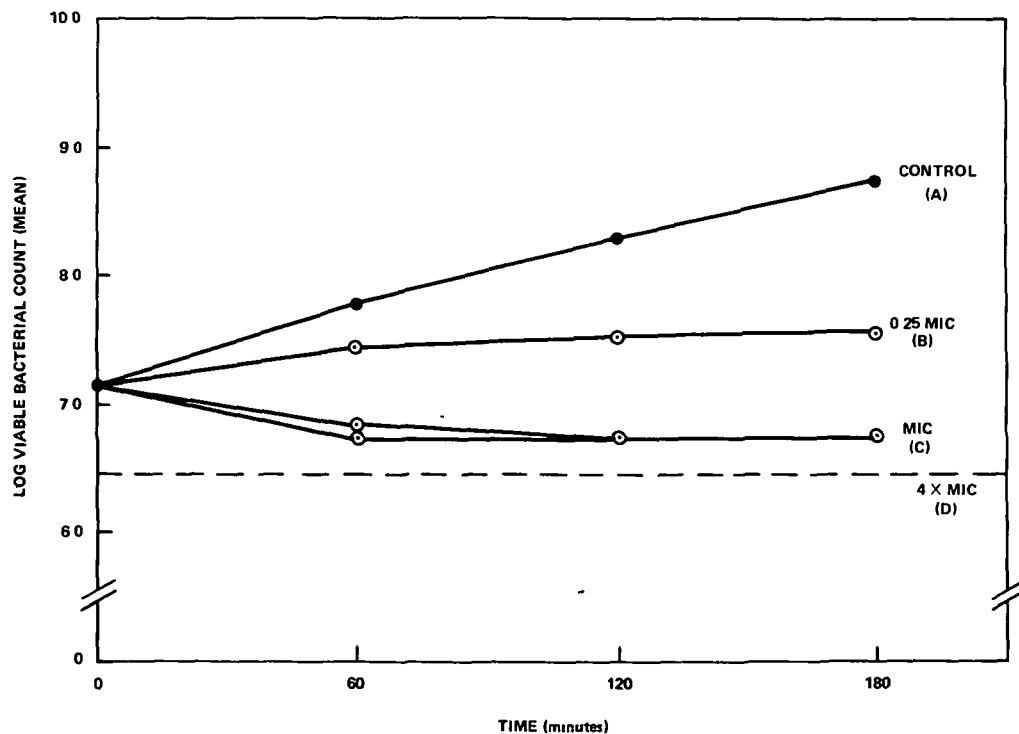


Figure 2-1. Change in bacterial ATP with time when bacteria are grown in the presence of gentamicin.

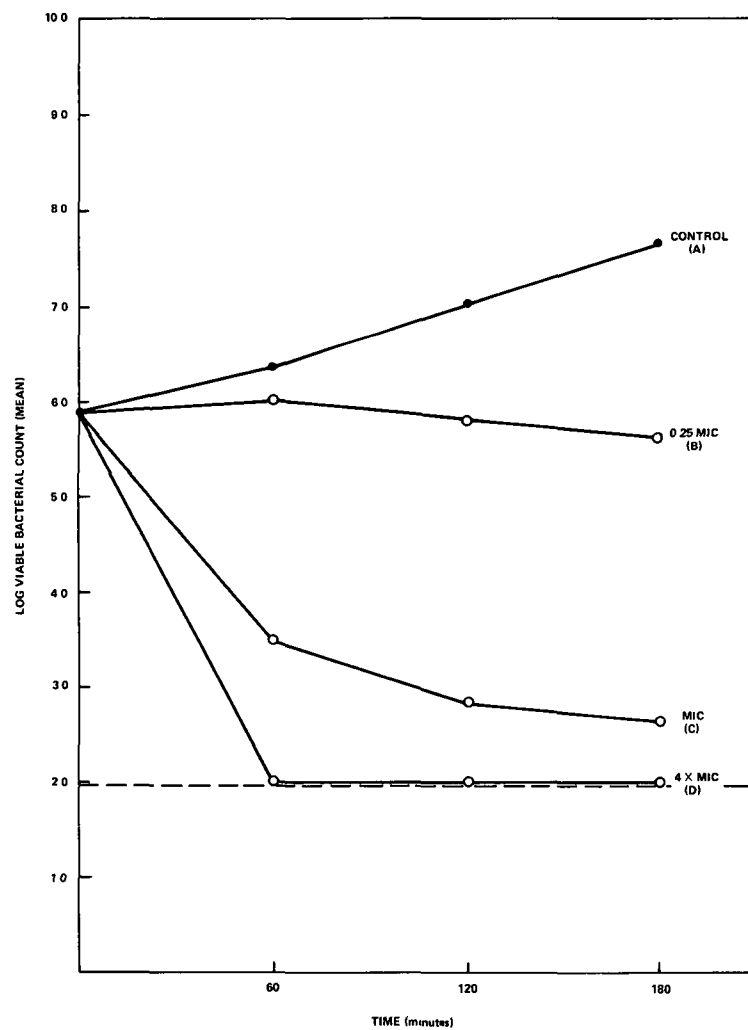


Figure 2-2. Change in colony counts with time when bacteria are grown in the presence of gentamicin.

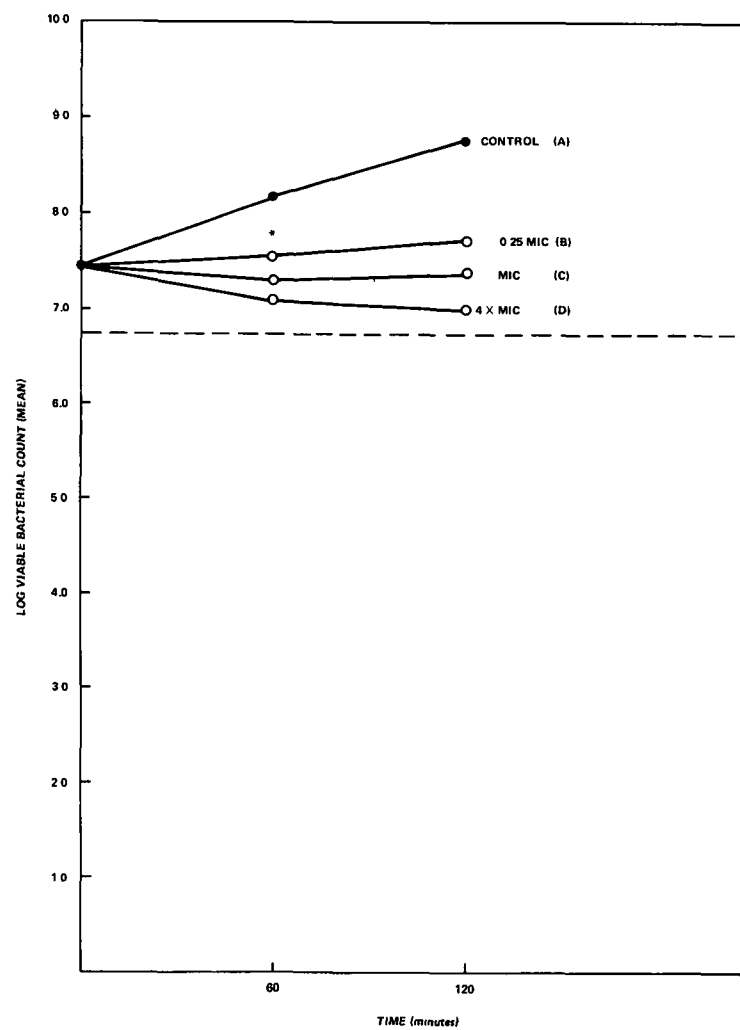


Figure 2-3. Change in bacterial ATP with time when bacteria are grown in the presence of tetracycline.

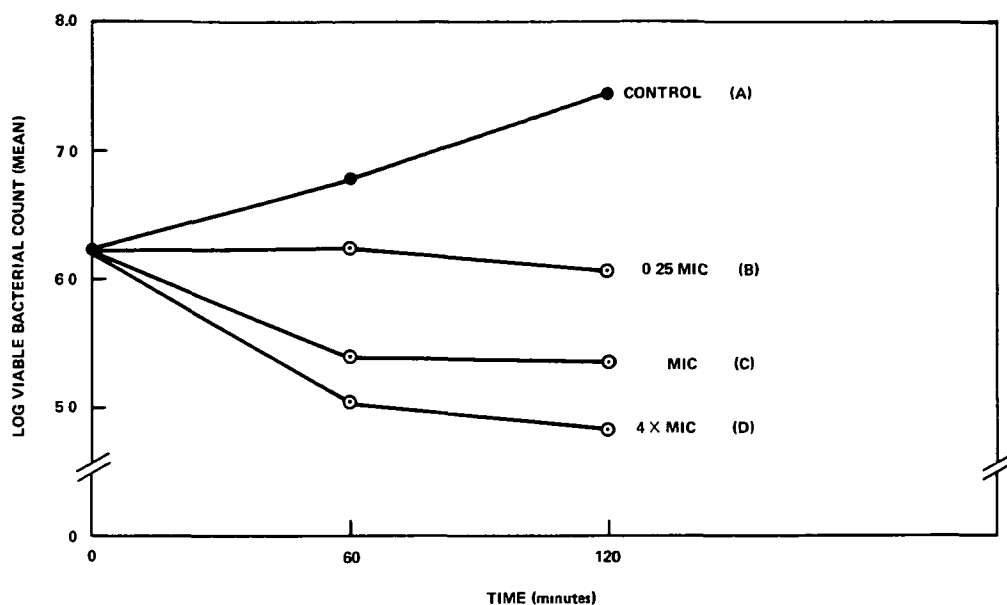


Figure 2-4. Change in colony counts with time when bacteria are grown in the presence of tetracycline.

With gentamicin, the effect of the MIC on viable colony counts was predictably different from the degree of inhibition due to 0.25 MIC. The inhibition of bacterial ATP at the MIC was such that blank level readings were generally obtained within 2 hours.

With tetracycline, the degree of inhibition of bacterial ATP was never great enough to approach blank levels nor was the broth ever sterilized. This clearly reflects the differing mode of action of these two antimicrobial drugs. (That is, gentamicin is bactericidal; tetracycline is bacteriostatic.)

The degree of inhibition was analyzed by measuring simple differences (that is, 0.5 or 1 log) between the control and the inhibited curves and by calculating the ratios between the slope of the inhibited curve and the control curve after 2 hours of log phase growth.

$$\frac{X_2 - A_0}{A_2 - A_0}$$

where X_2 = assay of inhibited curve after 2 hours

A_0 = assay of control at 0 hour

A_2 = assay of control at 2 hours

It was hoped that such calculations (see tables 2-1 through 2-4) would yield some fixed measurable index of inhibition that would correlate well with the tube dilution MIC. No such predictable index of inhibition could be determined by which we could state that achieving that end point meant that the MIC had been reached or exceeded.

Table 2-1
Change in Bacterial ATP (Log) When Bacteria Are Grown in the Presence of Gentamicin*

	A ₀	A ₁	B ₁	C ₁	D ₁	A ₂	B ₂	C ₂	D ₂	A ₃	B ₃	C ₃	D ₃
<i>E. coli</i>	6.63	7.33	7.05	6.56	6.42	8.05	6.93	6.32	6.32	8.73	6.57	6.34	6.37
Index =							+0.21	-0.22			-0.03	-0.14	
<i>Klebsiella</i>	7.61	8.44	8.13	6.90	6.51	8.87	8.39	6.65	6.55	9.21	8.49	6.56	6.57
Index =							+0.62	-0.76			+0.61	-0.66	
<i>Enterobacter</i>	7.16	7.94	6.97	6.47	6.44	8.60	6.81	6.44	6.44	9.03	6.66	6.38	6.42
Index =							-0.24	-0.5			-0.27	-0.42	
<i>P. mirabilis</i>	7.50	8.13	7.54	6.87	6.66	8.68	7.15	6.68	6.63	8.96	7.14	6.71	6.62
Index =							-0.29	-0.69			-0.25	-0.54	
<i>Pseudomonas</i>	7.03	7.57	7.38	6.58	6.58	8.08	7.52	6.56	6.55	8.35	7.72	6.65	6.65
Index =							+0.47	-0.45			+1.10	-0.29	
<i>S. aureus</i>	7.06	7.46	7.36	6.64	6.60	7.90	7.58	6.68	6.70	8.29	7.64	6.65	6.63
Index =							+0.62	-0.45			+0.47	-0.33	
<i>S. epidermidis</i>	6.84	6.97	6.97	6.79	6.78	7.31	7.16	6.60	6.73	7.76	7.43	6.72	6.61
Index =							+0.68	-0.51			+0.64	-0.13	
<i>Enterococcus</i>	7.39	8.28	8.15	8.10	7.97	8.74	8.61	8.09	7.95	9.39	8.78	8.18	8.02
Index =							+0.90	+0.52			+0.70	+0.40	
Mean	7.15	7.77	7.44	6.86	6.75	8.28	7.52	6.75	6.73	8.72	7.55	6.77	6.73
							±0.66	±0.55					

*Where A = control (no gentamicin); B = ¼ MIC; C = MIC; D = 4 × MIC.

A₀ = assay at time zero; A₁ = after growth for 1 hour; A₂ = after 2 hours; A₃ = after 3 hours.

Table 2-2
Change in Viable Colony Counts (Log) When Bacteria Are Grown in the Presence of Gentamicin

	A ₀	A ₁	B ₁	C ₁	D ₁	A ₂	B ₂	C ₂	D ₂	A ₃	B ₃	C ₃	D ₃
<i>E coli</i>	5.31	6.19	5.17	<2.0	<2.0	7.17	5.05	<2.0	<2.0	8.15	3.89	2.30	<2.0
Index =							-0.14	-1.78					
<i>Klebsiella</i>	6.38	6.88	6.84	2.85	<2.0	7.69	7.21	2.60	<2.0	8.36	7.37	3.78	<2.0
Index =							+0.63	-2.88					
<i>Enterobacter</i>	6.00	6.64	5.70	3.60	<2.0	7.60	4.90	3.48	<2.0	7.97	5.02	3.60	<2.0
Index =							-0.69	-1.58					
<i>P mirabilis</i>	6.45	7.00	6.26	2.98	<2.0	7.73	5.04	2.78	<2.0	8.39	5.28	2.00	<2.0
Index =							-1.10	-2.87					
<i>Pseudomonas</i>	6.06	6.13	6.12	3.58	<2.0	6.67	6.01	2.30	2.0	7.42	6.25	<2.0	<2.0
Index =							-0.08	-6.16					
<i>S aureus</i>	5.29	5.48	5.39	4.10	<2.0	5.86	5.36	2.48	<2.0	6.31	5.29	<2.0	<2.0
Index =							+0.12	-4.93					
<i>S epidermidis</i>	5.36	5.42	5.44	5.25	4.02	5.88	5.73	4.29	<2.0	6.40	6.07	3.43	2.85
Index =							+0.71	-2.06					
<i>Enterococcus</i>	6.37	7.18	7.04	3.64	<2.0	7.64	6.99	2.90	<2.0	8.29	5.67	2.0	<2.0
Index =							+0.49	-2.73					
Mean	5.90	6.37	6.00	3.50	<2.0	7.03	5.79	2.85	<2.0	7.66	5.61	2.64	<2.0
							-0.10	-2.70					

Table 2-3
Change in Bacterial ATP (Log) When Bacteria Are Grown in Presence of Tetracycline

	A ₀	A ₁	B ₁	C ₁	D ₁	A ₂	B ₂	C ₂	D ₂	Blank
<i>E. coli</i>	7.28	7.84	7.52	7.44	7.17	8.60	7.80	7.53	7.19	6.34
Index =							+0.39	+0.20		
<i>Klebsiella</i>	7.52	8.41	7.74	7.55	7.00	8.88	7.75	7.53	6.53	6.22
Index =							+0.17	-0.01		
<i>Enterobacter</i>	7.54	8.52	7.52	7.46	7.25	8.97	7.64	7.51	7.02	6.52
Index =							+0.07	-0.02		
<i>P. mirabilis</i>	7.71	8.46	7.38	6.57	6.46	8.93	7.36	6.54	6.44	6.37
Index =							-0.29	+0.96		
<i>Pseudomonas</i>										
<i>S. aureus</i>	7.10	7.40	7.24	7.08	7.02	7.97	7.53	7.23	7.12	6.41
Index =							+0.49	+0.15		
<i>S. epidermidis</i>										
<i>Enterococcus</i>	7.63	8.42	7.97	7.77	7.68	9.14	8.24	7.91	7.70	6.40
Index =							+0.40	+0.18		
Mean	7.46	8.18	7.56	7.31	7.10	8.75	7.72	7.38	7.00	6.38
							+0.20	-0.06		

Table 2-4
Change in Viable Colony Counts (Log) When Bacteria Are
Grown in Presence of Tetracycline

	A ₀	A ₁	B ₁	C ₁	D ₁	A ₂	B ₂	C ₂	D ₂
<i>E. coli</i>	6.02	6.57	6.11	5.95	5.09	7.57	6.42	5.59	4.16
Index							+0.26	-0.38	
<i>Klebsiella</i>	6.29	7.24	6.56	6.02	4.98	7.84	6.66	5.99	4.20
Index							+0.24	-0.19	
<i>Enterobacter</i>	6.36	6.98	6.37	6.29	6.18	7.70	6.35	6.24	5.88
Index							-0.01	-0.09	
<i>P. mirabilis</i>	6.56	7.16	6.12	<2.0	<2.0	8.00	4.65	2.30	2.70
Index							-1.33	-2.06	
<i>Pseudomonas</i>									
<i>S. aureus</i>	5.51	5.54	5.53	5.46	5.44	5.88	5.60	5.39	5.42
Index							+0.24	-0.32	
<i>S. epidermidis</i>									
<i>Enterococcus</i>	6.57	7.18	6.74	6.69	6.63	7.65	6.74	6.55	6.61
Index							+0.16	-0.02	
Mean	6.22	6.78	6.24	5.40	5.05	7.44	6.07	5.34	4.83
							-0.12	-1.01	

CONCLUSION

No fixed measurable index of inhibition could be determined which correlated well with the tube dilution MIC. This is not surprising because any attempt to correlate the effect of antimicrobial agents on the metabolic events in the bacterium in the first few hours of log phase growth with the ability of the drug to inhibit visible (that is, $> 10^7$ bacteria/ml) growth at 18 to 24 hours is likely to be futile.

ALTERNATIVE APPROACHES TO THE PROBLEM

One approach would be to utilize some predetermined index of inhibition and compare the concentration of the drug required to achieve this effect with the tube dilution MIC. This approach has been used to validate the radiometric method of measuring antibiotic effect on bacterial growth by DeBlanc et al., 1972. Their analysis resulted in a complex table of dilution factors that varied from antibiotic to antibiotic versus the same microorganism. Their results therefore are frankly unconvincing.

Another approach would be to utilize some predetermined index of inhibition and attempt to validate it as a more accurate and clinically more relevant index of antimicrobial drug sensitivity testing than the MIC. Considering the degree to which the MIC method and its derivatives (namely the Kirby-Bauer agar-diffusion technique) are entrenched in the medical community and literature, this would appear to be a gargantuan task beyond the present scope of the study.

A third approach might be the development of a statistical-mathematical model on which we can analyze our present data to achieve the desired end. This would require professional consultation.

The most expedient approach, however, would be to model our validation on the method described by Isenberg et al. (1971). This involves correlating the results of the inhibition of bacterial ATP with the broad categories of "sensitive," "resistant," and "intermediate" which are yielded by the Kirby-Bauer technique.

Although rigorous evaluation of the Kirby-Bauer single disc diffusion method is usually performed by plotting regression graphs comparing agar dilution MIC's and disc diffusion zone diameters (Matsen et al., 1970; Ericsson and Sherris, 1971), nevertheless, the breakpoints can be determined without necessarily demonstrating a direct linear regression curve. In fact, often there is a distinctly bimodal distribution of points along these regression curves and certainly as practiced, the zone diameters cannot be used to predict the precise MIC.

This proposal has been examined in a preliminary way by measuring the index of inhibition (slope-ratio method) of tetracycline on five bacterial species. The concentrations of tetracycline used were those at the upper (16 $\mu\text{g/ml}$) and lower (4 $\mu\text{g/ml}$) limits of breakpoint MIC's (see table 2-5). These results were then compared with the Kirby-Bauer categories. In each instance, at 4 $\mu\text{g/ml}$ of tetracycline, a sensitive organism yielded a negative index of inhibition and a resistant microorganism resulted in a positive index. This resolution was

clearly evident after only 1 hour of log phase growth. At 16 µg/ml of tetracycline, the resistant *proteus* yielded a negative index at 1 hour, but this was positive again at 2 hours.

These encouraging results will cause adoption of this mode of evaluation. This will entail carrying out the Kirby-Bauer agar diffusion techniques rigorously on all isolates.

Table 2-5
Zone-size Interpretive Standards and Approximate MIC
Breakpoints for Disc Diffusion Testing

Antimicrobial agent	Disc potency	Inhibitory zone diameter (to nearest mm)			Approx. MIC breakpoint	
		Resistant	Intermediate	Susceptible	Resistant	Susceptible
Penicillin G	10 U (6.3 µg)					
Ampicillin	10 µg					
<i>Staphylococci</i>		20 or less	21-28	29 or more	Penase*	≤ 0.1 µg/ml
<i>Enterobacteriaceae</i>		11 or less	12-13	14 or more	≥ 32 µg/ml	≤ 5-15 µg/ml†
& <i>enterococci</i>		11 or less	12-21	22 or more	≥ 32 µg/ml	≤ 1.5 µg/ml
Other organisms						
Methicillin	5 µg	9 or less	10-13	14 or more		≤ 2.5 µg/ml
Nafcillin or						
Oxacillin	1 µg	10 or less	11-12	13 or more		≤ 0.6 µg/ml
Vancomycin	30 µg	9 or less	10-11	12 or more		≤ 5 µg/ml
Cephalothin	30 µg	14 or less	15-17	18 or more	≥ 32 µg/ml	≤ 10 µg/ml
Cephaloridine	30 µg	11 or less	12-15	16 or more	≥ 40 µg/ml	≤ 10 µg/ml
Carbenicillin	50 µg					
<i>Pseudomonas</i> sp.		12 or less	13-14	15 or more	≥ 250 µg/ml	≤ 125 µg/ml
<i>Proteus</i> & <i>E. coli</i>		17 or less	18-22	23 or more	≥ 32 µg/ml	≤ 16 µg/ml
Polymyxin B ‡	300 U (30 µg)	10 or less	11-14	15 or more	mean ≤ 12.5 U/ml	
Chloramphenicol	30 µg	12 or less	13-17	18 or more	≥ 25 µg/ml	≤ 12.5 µg/ml
Tetracycline	30 µg	14 or less	15-18	19 or more	≥ 12.5 µg/ml	≤ 4 µg/ml
Erythromycin	15 µg	13 or less	14-17	18 or more	≥ 8 µg/ml	≤ 2 µg/ml
Lincomycin	2 µg	9 or less	10-14	15 or more	≥ 8 µg/ml	≤ 2 µg/ml
Clindamycin	2 µg	11 or less	12-15	16 or more	≥ 8 µg/ml	≤ 2 µg/ml
Kanamycin	30 µg	13 or less	14-17	18 or more	≥ 25 µg/ml	≤ 6 µg/ml
Neomycin	30 µg	12 or less	13-16	17 or more		≤ 10 µg/ml
Streptomycin	10 µg	11 or less	12-14	15 or more	≥ 15 µg/ml	≤ 6 µg/ml
Gentamicin	10 µg	12 or less	13-14	15 or more	≥ 12.5 µg/ml	≤ 6 µg/ml
Sulfonamides §	300 µg	12 or less	13-16	17 or more	≥ 35 mg%	≤ 10 mg%
Nitrofurantoin	300 µg	14 or less	15-18	19 or more	≥ 100 µg/ml	≤ 25 µg/ml
Nalidixic Acid	30 µg	13 or less	14-18	19 or more	≥ 12.5 µg/ml	≤ 12.5 µg/ml

*Penicillinase-producing staphylococci.

†MIC dependent upon dilution method used.

‡Polymyxin B diffused poorly in agar and the accuracy of the diffusion method is less than with other antimicrobics.

Resistance is always significant, but when treatment of systemic infections due to susceptible strains is considered, it is wise to confirm the results of a diffusion test with a dilution method.

§300 µg or 250 µg sulfonamide disc can be used with the same standards of zone interpretation (MIC values are for sulfamethizole).

||Urinary tract infections only.

TECHNICAL MODIFICATIONS

During the initial phase of study, several procedural changes have occurred. Simultaneous viable colony counts will no longer be performed because they are very time-consuming and do not appear to yield substantive data relevant to the present purpose. The frozen glass bead method of storage of stock cultures has proven somewhat disappointing, as several of the organisms do not yield heavy broth cultures after overnight incubation. Agar slants will be used. In order to increase the yield of work, both the DuPont Luminescence 760 Biometer and the Aminco Chem-Glow photometer will be used to assay ATP (Nibley and Thomas, 1976). At the present initial inoculum size ($\sim 10^6$ viable organisms/ml), a concentrating procedure (that is, centrifugation) is not necessary.

The source of the apparent discrepancy between the viable colony counts and the conversion of bacterial ATP to numbers of bacteria has not yet been determined. This one log difference is not due to error in preparing ATP standards, nor is it related to differences in instrumentation. It most likely reflects procedural changes in the method of assay of bacterial ATP, possibly the omission of the malic-arsenate buffer step.

SECTION 3

INTERIM PROGRESS REPORT, AUGUST 1973: CORRELATION WITH STANDARD KIRBY-BAUER METHODOLOGY

INTRODUCTION

Because preliminary results of the protocol precluded correlation with tube-dilution MIC's, as described in the previous section, the correlation of the results of the inhibition of bacterial ATP with the broad categories of "sensitive," "resistant," and "intermediate" designated by the Kirby-Bauer agar diffusion technique was attempted.

METHODS

The Kirby-Bauer agar diffusion technique was carried out exactly according to the standards recommended by the National Committee for Clinical Laboratory Standards in August 1972.

Inhibition of bacterial ATP was generally measured at two antibiotic concentrations approximating the MIC breakpoints. This will help to determine the single antibiotic concentration which results in the best discrimination. Samples were assayed at 0 hour (that is, after a 1-hour preincubation period) and after 1 and 2 hours of log phase growth. The degree of inhibition was quantitated by the slope-ratio method. The tentative interpretation of the Index of Inhibition is as follows:

<u>ATP INDEX</u>	<u>INTERPRETATION</u>
$\geq + 0.25$	Resistant
0.15 to 0.24	Intermediate
< 0.15	Sensitive

RESULTS AND DISCUSSION

β -Lactam Antibiotics

When a β -lactam antibiotic is added to a sensitive gram-negative bacterium in the logarithmic phase of growth, the bacterium undergoes a series of morphologic changes from filamentous forms to bulbous or "bow-tie" forms to spheroplasts before finally undergoing osmotic lysis (Greenwood and O'Grady, 1973). These changes are related to drug-induced alterations in the cell-wall structure and may be dependent on the relative inhibitory effect of β -lactam antibiotics on two different enzymes: an endopeptidase concerned with cell division and a glycosidase concerned with cell growth (Hartmann et al., 1972). The rates at which these changes occur are dependent on the concentration of the drug, the type and rate of production of β -lactamase by the bacterium, and the stability of the drug to penicillinase. Also, the time to lysis is dependent on the osmolality of the medium (being more rapid at lower

osmolalities) and the osmotic susceptibility of the species with damaged cell walls (*E. Coli* being more susceptible than *P. mirabilis*). Continuous turbidometric recordings of these events show increasing opacification of the culture (despite lack of cell division) for varying lengths of time after the addition of the drug until lysis occurs (Greenwood and O'Grady, 1973).

Conventional susceptibility testing requiring overnight incubation merely reflects the final result of this complex series of responses. When bacterial ATP is used as a measure of β -lactam antibiotic effect on log phase cultures, it is apparent that the drop in bacterial ATP (indicating sensitivity) coincides with the time to achieve lysis. This time to achieve lysis has varied from less than 1 hour to greater than 6 hours. Accordingly, this delay restricts the rapidity by which sensitivity can be determined by the ATP technique. As well, the addition of Triton X-100 in the assay procedure will enhance osmotic lysis of these cell-wall-deficient bacteria.

Penicillin G

Bacterial susceptibility to penicillin G (see tables 3-1, 3-2, 3-3, and 3-4) is a rather complex problem. Strains of *S. aureus* need special consideration because those with MIC's of over 0.1 $\mu\text{g/ml}$ are almost invariably penicillinase producers and by definition must be reported as resistant. Both a penicillin-sensitive *S. aureus* (ATCC 25923) and a penicillin-resistant *S. aureus* (S-187) were evaluated at 0.15 $\mu\text{g/ml}$ and discrimination was possible at 2 hours (table 3-4).

Clinical experience has shown that *enterococci*, *P. mirabilis*, and some strains of *E. coli* with MIC's up to 128 $\mu\text{g/ml}$ respond to penicillin—especially in urinary tract infections. Accordingly, the sensitivity data at 128 $\mu\text{g/ml}$ in table 3-1 must be interpreted in relation to the MIC's. Comparison with the Kirby-Bauer technique is not valid.

In view of these factors, it is difficult to choose a single concentration of penicillin G, because choosing the higher concentrations applicable to urinary tract infections might lead to incorrect interpretation of *S. aureus* sensitivity.

The time to lysis was studied with *E. coli* and penicillin G (figure 3-1) and shown to be about 2 hours. The effect of Triton X-100 was measured and shown to be negligible on the control culture, but there is a population of penicillin G-induced cell-wall-deficient organisms which are susceptible to Triton X-100 lysis. This latter effect did not alter the interpretation of the sensitivity test.

Ampicillin

The correlation with the Kirby-Bauer technique (see tables 3-5 and 3-6) was good in the 8 to 16 $\mu\text{g/ml}$ range except for *P. mirabilis*, which was consistently falsely-resistant. The explanation for this lies in the fact that the time to lysis with this particular combination is 3 hours (figure 3-2).

Table 3-1
Bacterial Susceptibility to Penicillin G

Microorganism	K-B Zone Diameter (mm)	Tube Dilution MIC ($\mu\text{g/ml}$)	0.25 $\mu\text{g/ml}$				128 $\mu\text{g/ml}$			
			ATP Index		Interpretation		ATP Index		Interpretation	
			1 hr.	2 hr.	K-B	ATP Index	1 hr.	2 hr.	K-B	ATP Index
<i>E coli</i>	-	128	+1.09	+0.96	R	R	+0.68	-0.69	R	R $\rightarrow$ S
<i>Klebsiella</i>	-	>128	+0.97	+1.01	R	R	+1.00	+0.89	R	R
<i>Enterobacter</i>	-	>128	+1.05	+0.89	R	R	+1.24	+1.01	R	R
<i>P mirabilis</i>	20	4	+1.00	+0.99	I	R	+0.23	+0.25	I	I
<i>Pseudomonas</i>	-	>128	+1.10	+1.02	R	R	+0.92	+0.92	R	R
<i>S aureus</i>	32.5	<0.125	-0.55	-0.72	S	S	-0.73	-0.07	S	S
<i>S epidermidis</i>	21	1	+0.09	+0.47	I	S $\rightarrow$ R	-0.55	-1.38	I	S
<i>Enterococcus</i>	18	4	+1.00	+0.96	I	R	-0.82	-0.42	I	S

Table 3-2
Bacterial Susceptibility to Penicillin G
at 0.25 $\mu\text{g/ml}$ (B) and 128 $\mu\text{g/ml}$ (C)

	A ₀	B ₀	C ₀	A ₁	B ₁	C ₁	A ₂	B ₂	C ₂
<i>E. coli</i> ATP (fg/ml)	8.85×10^6	9.75×10^6	9.45×10^6	3.24×10^7	3.68×10^7	2.14×10^7	1.66×10^8	1.50×10^8	1.26×10^6
bacteria/ml	3.20×10^7	3.53×10^7	3.72×10^7	1.17×10^8	1.73×10^8	7.76×10^7	6.01×10^8	5.41×10^8	4.55×10^6
log bacteria/ml	7.51	7.55	7.53	8.07	8.12	7.89	8.78	8.73	6.66
Index					+1.09	+0.68		+0.96	-0.67
<i>Klebsiella</i> ATP (fg/ml)	1.15×10^7	1.35×10^7	1.28×10^7	6.50×10^7	6.10×10^7	6.50×10^7	2.28×10^8	2.32×10^8	1.65×10^8
bacteria/ml	4.18×10^7	4.91×10^7	4.64×10^7	2.36×10^8	2.22×10^8	2.36×10^8	8.27×10^8	8.44×10^8	6.00×10^8
log bacteria/ml	7.62	7.69	7.67	8.37	8.35	8.37	8.92	8.93	8.78
Index					+0.97	+1.00		+1.01	+0.89
<i>Enterobacter</i> ATP (fg/ml)	8.80×10^6	8.45×10^6	8.95×10^6	2.15×10^7	2.23×10^7	2.62×10^7	7.85×10^7	6.35×10^7	8.15×10^7
bacteria/ml	3.20×10^7	3.07×10^6	3.25×10^6	7.82×10^7	8.11×10^7	9.53×10^7	2.85×10^8	2.31×10^8	7.96×10^8
log bacteria/ml	7.51	7.49	7.51	7.89	7.91	7.98	8.46	8.36	8.47
Index					+1.05	+1.24		+0.89	+1.01
<i>S. aureus</i> ATP (fg/ml)	2.36×10^6	2.67×10^6	2.67×10^6	3.94×10^6	1.80×10^6	1.61×10^6	9.45×10^6	8.80×10^5	6.18×10^5
bacteria/ml	8.58×10^6	9.69×10^6	9.69×10^6	1.42×10^7	6.90×10^6	5.83×10^6	3.42×10^7	3.19×10^6	2.23×10^6
log bacteria/ml	6.93	6.99	6.99	7.15	6.81	6.77	7.53	6.50	6.35
Index					-0.55	-0.73		-0.72	-0.97
<i>S. epidermidis</i> ATP (fg/ml)	3.16×10^6	3.24×10^6	3.39×10^6	6.85×10^6	3.42×10^6	2.10×10^6	1.52×10^7	6.63×10^6	3.60×10^5
bacteria/ml	1.15×10^7	1.11×10^7	1.23×10^7	2.48×10^7	1.24×10^7	7.58×10^6	5.48×10^7	2.40×10^7	1.20×10^6
log bacteria/ml	7.06	7.07	7.09	7.39	7.09	6.88	7.74	7.38	6.12
Index					+0.09	-0.55		+0.47	-1.38
<i>Enterococcus</i> ATP (fg/ml)	1.10×10^7	1.04×10^7	1.20×10^7	4.07×10^7	4.11×10^7	3.73×10^6	1.77×10^8	1.60×10^8	3.40×10^6
bacteria/ml	3.98×10^7	3.75×10^7	4.34×10^7	1.17×10^8	1.49×10^8	1.35×10^7	6.39×10^8	5.79×10^8	1.23×10^7
log bacteria/ml	7.60	7.57	7.64	8.17	8.17	7.13	8.81	8.76	7.09
Index					+1.00	-0.82		+0.96	-0.42
<i>P. mirabilis</i> ATP (fg/ml)	4.57×10^6	4.34×10^6	4.91×10^6	1.65×10^7	1.15×10^7	6.25×10^6	7.20×10^7	7.10×10^7	9.20×10^6
bacteria/ml	1.65×10^7	1.57×10^7	1.78×10^7	5.97×10^7	5.97×10^7	2.26×10^7	2.61×10^8	2.57×10^8	3.33×10^7
log bacteria/ml	7.22	7.20	7.25	7.78	7.78	7.35	8.42	8.41	7.52
Index					+1.00	+0.23		+0.99	+0.25
<i>Pseudomonas</i> ATP (fg/ml)	3.60×10^6	3.39×10^6	3.05×10^6	8.95×10^6	9.75×10^6	8.25×10^6	2.91×10^7	2.93×10^7	2.95×10^7
bacteria/ml	1.30×10^7	1.23×10^7	1.17×10^7	3.25×10^7	3.55×10^7	3.00×10^7	1.06×10^8	1.09×10^8	8.89×10^7
log bacteria/ml	7.12	7.09	7.07	7.51	7.55	7.48	8.02	8.04	7.95
Index					+1.10	+0.92		+1.02	+0.92

Table 3-3
E. coli (ATCC 25922) Susceptibility to
 Penicillin G at 128 µg/ml with and without TX

Specimen	A ₀	B ₀	A ₁	B ₁	A ₂	B ₂	A ₃	B ₃	A ₆	B ₆	A ₂₄	B ₂₄
Dilution Read	3		3	3	4	1	5	1	6	1	6	9
Plate (1)	26		51	12	23	26	17	13	15	3	39	32
(2)	21		45	9	28	22	20	9	14	2	32	34
(3)	29		59	13	34	22	16	16	17	1	40	33
(4)	25		42	20	34	32	11	15	17	4	44	33
Total Colonies	101		197	54	119	102	65	53	63	11	155	132
Viable col/ml	1.01 x 10 ⁶		1.97 x 10 ⁶	5.40 x 10 ⁵	1.19 x 10 ⁷	1.02 x 10 ⁴	6.50 x 10 ⁷	5.30 x 10 ³	6.30 x 10 ⁸	1.10 x 10 ³	1.55 x 10 ⁹	1.32 x 10 ⁵
log viable col/ml	6.00		6.29	5.73	7.08	4.01	7.81	3.72	8.80	3.04	9.19	5.12
<u>With Tx</u>												
ATP (fg/ml)	7.00 x 10 ⁶	7.95 x 10 ⁶	2.24 x 10 ⁷	1.70 x 10 ⁷	9.25 x 10 ⁷	9.10 x 10 ⁵	4.69 x 10 ⁸	8.65 x 10 ⁵	1.05 x 10 ⁹	4.85 x 10 ⁵	1.71 x 10 ⁹	2.83 x 10 ⁵
bacteria/ml	2.55 x 10 ⁷	2.89 x 10 ⁷	8.13 x 10 ⁷	6.16 x 10 ⁷	3.36 x 10 ⁸	3.31 x 10 ⁶	1.71 x 10 ⁹	3.15 x 10 ⁶	3.82 x 10 ⁹	1.76 x 10 ⁶	6.20 x 10 ⁹	1.03 x 10 ⁶
log bact/ml	7.41	7.46	7.91	7.79	8.53	6.52	9.23	6.50	9.58	6.25	9.79	6.01
Index				+0.76		-0.79		-0.50		-0.53		-0.59
<u>Without Tx</u>												
ATP (fg/ml)	7.30 x 10 ⁶	8.40 x 10 ⁶	2.41 x 10 ⁷	1.79 x 10 ⁷	1.28 x 10 ⁸	3.32 x 10 ⁶	5.95 x 10 ⁸	2.45 x 10 ⁶	1.01 x 10 ⁹	8.80 x 10 ⁵	1.67 x 10 ⁹	4.16 x 10 ⁵
bacteria/ml	2.65 x 10 ⁷	3.05 x 10 ⁷	8.76 x 10 ⁷	7.49 x 10 ⁷	4.64 x 10 ⁸	1.21 x 10 ⁷	2.16 x 10 ⁹	8.89 x 10 ⁶	3.65 x 10 ⁹	3.20 x 10 ⁶	6.05 x 10 ⁹	1.51 x 10 ⁶
log bact/ml	7.42	7.48	7.94	7.81	8.67	7.08	9.34	6.95	9.56	6.51	9.78	6.18
Index				+0.75		-0.27		-0.24		-0.43		-0.53

Table 3-4
S. aureus Susceptibility to Nafcillin and Penicillin

Microorganism	K-B (1 µg) Zone Diameter (mm)	Tube Dilution MIC (µg/ml)	Nafcillin (0.6 µg/ml)			
			ATP Index		Interpretation	
			1 hr.	2 hr.	K-B	ATP Index
<i>S. aureus</i> ATCC 25923	19.5	0.25	+0.64	+0.79	S	R → S
<i>S. aureus</i> S-187	19.5	0.25	+0.54	-0.39	S	R → S
			Penicillin (0.15 µg/ml)			
<i>S. aureus</i> ATCC 25923	32.5	<0.125	+0.36	-0.70	S	R → S
<i>S. aureus</i> S-187	14.5	N/D	+0.42	+0.53	R	R

Table 3-5
Bacterial Susceptibility to Ampicillin

Microorganism	K-B Zone Diameter (mm)	Tube Dilution MIC ($\mu\text{g/ml}$)	2 $\mu\text{g/ml}$				8 $\mu\text{g/ml}$			
			ATP Index		Interpretation		ATP Index		Interpretation	
			1 hr.	2 hr.	K-B	ATP Index	1 hr.	2 hr.	K-B	ATP Index
<i>E coli</i>	19	8	+1.04	+1.00	S	R	-0.75	-0.91	S	S
<i>Klebsiella</i>	0	>128	+1.08	+1.02	R	R	+0.93	+1.01	R	R
<i>Enterobacter</i>	0	>128	+1.02	+0.96	R	R	+1.07	+0.22	R	I
							16 $\mu\text{g/ml}$			
<i>P mirabilis</i> *	26.5	1	+0.99	+0.43	S	R	+0.80	+0.50	S	R
<i>Pseudomonas</i>	0	>128	+0.77	+0.99	R	R	+1.09	+1.03	R	R
<i>S aureus</i>	31	< 0.125	-0.87	-0.57	S	S	-0.84	-0.57	S	S
<i>S epidermidis</i>	25	0.5	+0.11	-0.31	S	S	+0.19	-0.03	S	S
<i>Enterococcus</i>	22	1	-0.36	-0.71	S	S	-0.82	-0.50	S	S

*Done in duplicate.

Table 3-6
P. mirabilis Susceptibility to Ampicillin
 at 2 µg/ml (B) and 16 µg/ml (C)

Specimen	A ₀	B ₀	C ₀	A ₁	B ₁	C ₁	A ₂	B ₂	C ₂	A ₃	B ₃	C ₃	A ₄	B ₄
Dilution Read	3			4	3	3	5	3	2	5	2	2	6	1
Plate 1	60			21	41	49	15	42	25	36	29	10	10	28
2	75			18	59	39	3	29	25	43	22	11	17	33
3	62			20	52	41	17	31	36	36	21	12	10	35
4	64			16	53	43	13	32	36	36	22	13	9	33
Total Colonies	261			75	205	172	48	134	122	151	94	46	46	129
Viable col/ml	2.61 x 10 ⁶			7.5 x 10 ⁶	2.05 x 10 ⁶	1.72 x 10 ⁶	4.8 x 10 ⁷	1.34 x 10 ⁶	1.22 x 10 ⁵	1.51 x 10 ⁸	9.4 x 10 ⁴	4.6 x 10 ⁴	4.6 x 10 ⁸	1.29 x 10 ⁴
log viable col/ml	6.42			6.88	6.31	6.24	7.68	6.13	5.09	8.18	4.97	4.66	8.66	4.11
ATP (fg/ml)	7.10 x 10 ⁶	8.18 x 10 ⁶	9.25 x 10 ⁶	3.45 x 10 ⁷	4.03 x 10 ⁷	2.55 x 10 ⁷	2.26 x 10 ⁸	6.05 x 10 ⁷	6.05 x 10 ⁷	4.21 x 10 ⁸	3.13 x 10 ⁶	4.20 x 10 ⁶	6.95 x 10 ⁸	3.24 x 10 ⁶
bacteria/ml	2.58 x 10 ⁷	2.97 x 10 ⁷	3.36 x 10 ⁷	1.25 x 10 ⁸	1.46 x 10 ⁸	9.28 x 10 ⁷	8.21 x 10 ⁸	2.20 x 10 ⁸	2.20 x 10 ⁸	1.53 x 10 ⁹	1.14 x 10 ⁷	1.53 x 10 ⁷	2.53 x 10 ⁹	1.18 x 10 ⁷
log bact/ml	7.41	7.47	7.53	8.10	8.17	7.97	8.91	8.34	8.34	9.18	7.06	7.18	9.40	7.07
Index					+1.10	+0.81		+0.62	+0.62		-0.20	-0.13		-0.17

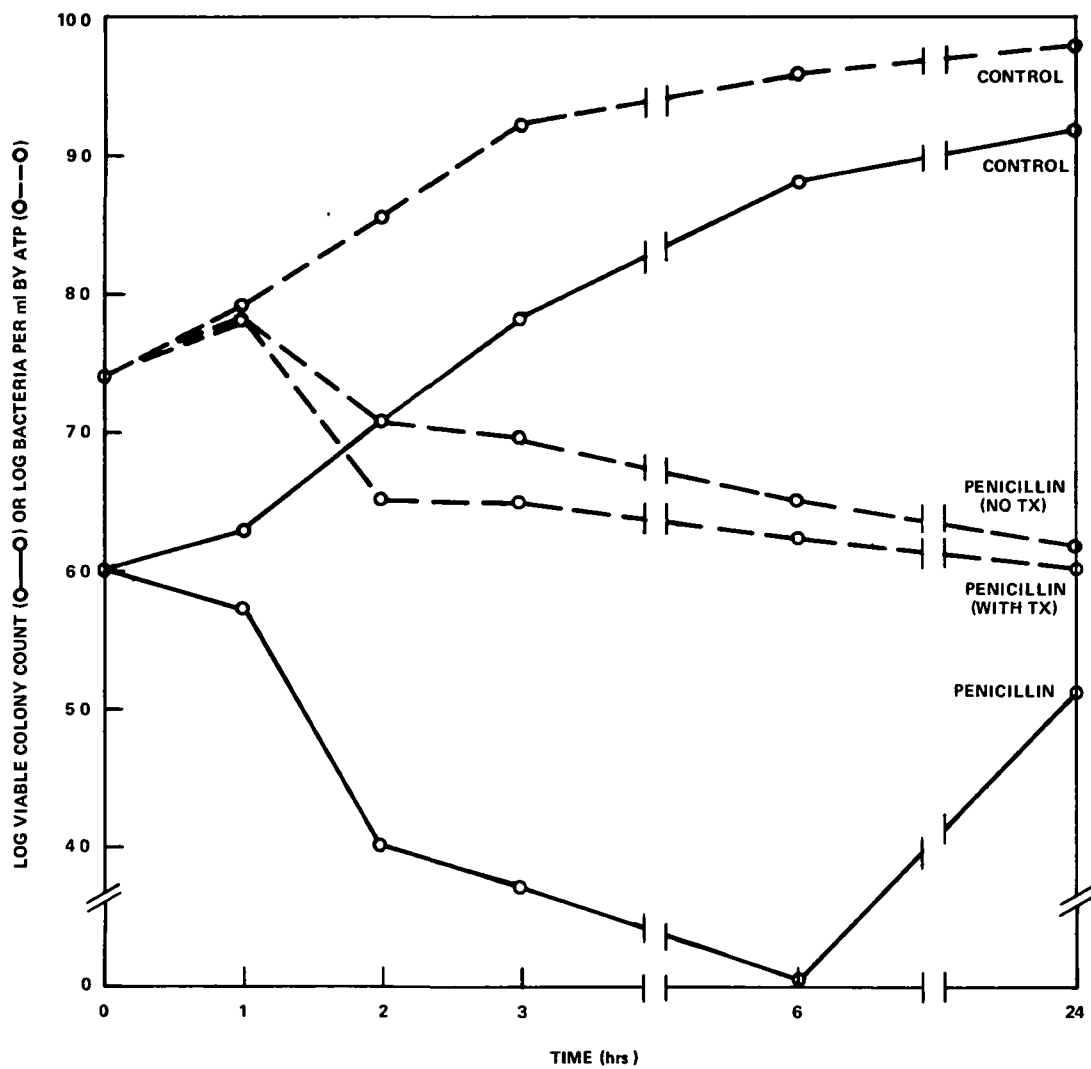


Figure 3-1. Time to lysis of *E. coli* grown in the presence of penicillin (128 µg/ml) with and without TX.

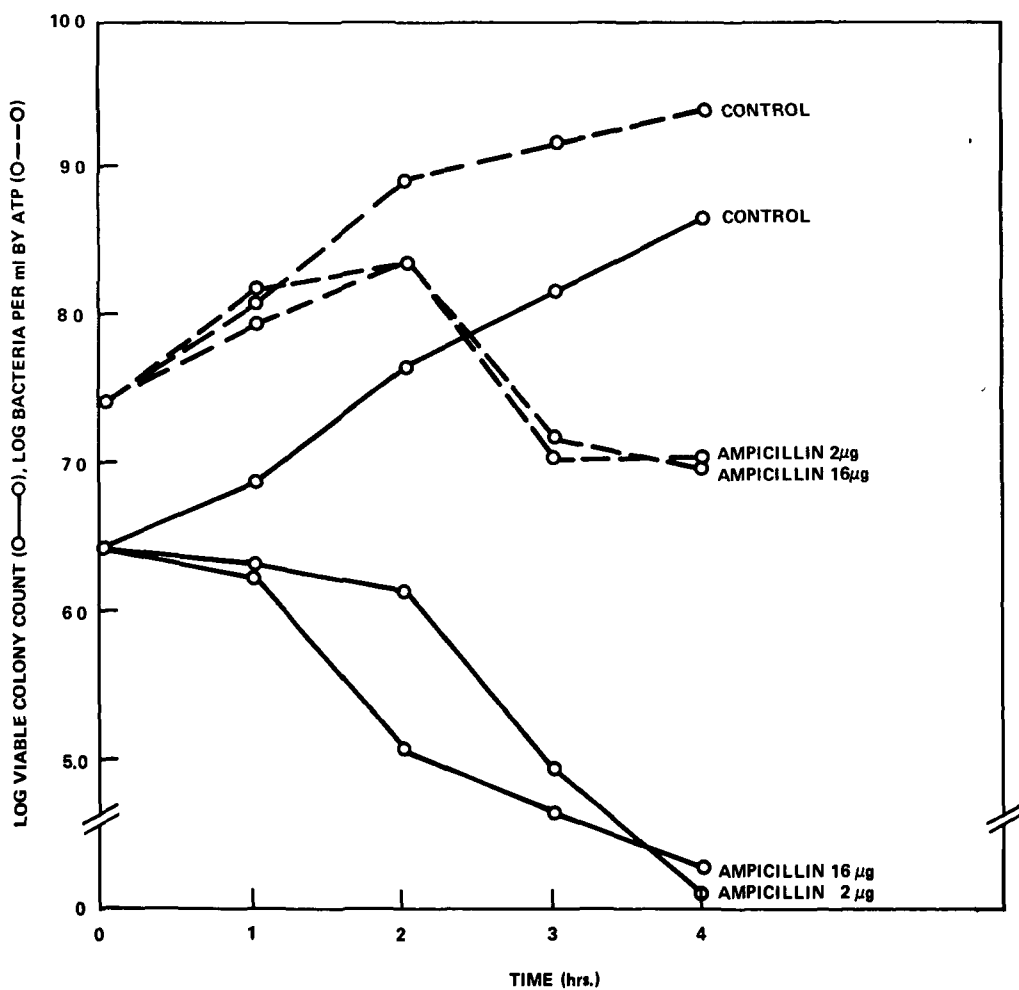


Figure 3-2. Time to lysis: *P. mirabilis* grown in the presence of ampicillin.

Nafcillin

The use of nafcillin (see tables 3-4 and 3-7) is limited to penicillinase-producing *S. aureus*, so the evaluation of this drug was restricted to a penicillin-sensitive (ATCC 25923) and a penicillin-resistant (S-187) strain of *S. aureus*. Only one antibiotic concentration was used because higher values imply resistance. The ability of the ATP technique to discriminate between these two strains was complete at 2 hours.

Carbenicillin

Although carbenicillin (see tables 3-8, 3-9, and 3-10) is of limited usefulness, we included it in our study because of the recent availability of an oral form, Indanyl Carbenicillin, recommended for urinary tract infections. Disc-sensitivity data are available only for *Pseudomonas*, *E. coli*, and *Proteus*, so comparison is restricted to these species (Schoenknecht, 1973). The MIC breakpoints also differ between these species adding additional complexity, although bacteria inhibited by concentrations achieved in the urine should be considered sensitive to this agent.

The correlation is complete with *E. coli* and *Proteus* at 2 hours, but the *Pseudomonas* is falsely-resistant. A more detailed study of this (figure 3-3) demonstrated a markedly prolonged time to lysis of between 6 and 24 hours, making "rapid" sensitivity testing by this method impossible.

Cephalothin

The correlation with cephalothin (table 3-11) is excellent at the higher concentration (32 $\mu\text{g/ml}$). The apparent discrepancy with the *enterococcus* is not bothersome since the MIC of 32 $\mu\text{g/ml}$ is borderline and does not correlate well with the disc-sensitivity method. It is worth emphasizing that the ATP index may in certain instances correlate better with the MIC than the disc-sensitivity result and that these should not necessarily be considered as discordant results.

Tetracycline

At 4 $\mu\text{g/ml}$, the correlation with tetracycline (see table 3-12) is 100 percent except for *Pseudomonas* where the index falls into the intermediate range at 2 hours. We have not studied this in more detail. Both the *Proteus* and *Pseudomonas* are falsely-sensitive at 16 $\mu\text{g/ml}$. Therefore the final concentration chosen should be $\sim 6 \mu\text{g/ml}$.

Erythromycin

Although erythromycin (tables 3-13 and 3-14) generally has limited usefulness in the treatment of urinary tract infections due to its limited antibacterial spectrum, there are studies (Zinner et al., 1971) to indicate that alkalinization of the urine increases this drug's activity and antibacterial spectrum to include most gram-negative bacilli. The correlation between the Kirby-Bauer and the ATP index is 100 percent at 1 and 2 hours.

Table 3-7
S. aureus Susceptibility to Nafcillin
 at 0.6 µg/ml (B) and to Penicillin G at 0.15 µg/ml (C)

	A ₀	B ₀	C ₀	A ₁	B ₁	C ₁	A ₂	B ₂	C ₂
<i>S. aureus</i> ATCC 25923									
ATP (fg/ml)	3.04×10^6	3.42×10^6	3.28×10^6	3.90×10^6	3.51×10^6	3.32×10^6	6.50×10^6	1.67×10^6	1.77×10^6
bacteria/ml	1.11×10^7	1.24×10^7	1.19×10^7	1.42×10^7	1.28×10^7	1.21×10^7	2.36×10^7	6.07×10^6	6.44×10^6
log bacteria/ml	7.04	7.09	7.08	7.15	7.11	7.08	7.37	6.78	6.81
Index					+0.64	+0.36		-0.79	-0.70
<i>S. aureus</i> S-187									
ATP (fg/ml)	5.85×10^6	4.75×10^6	4.55×10^6	1.07×10^7	8.05×10^6	7.60×10^6	2.30×10^7	3.50×10^6	1.19×10^7
bacteria/ml	2.13×10^7	1.73×10^7	1.65×10^7	3.87×10^7	2.93×10^7	2.76×10^7	8.35×10^7	1.27×10^7	4.33×10^7
log bacteria/ml	7.33	7.24	7.22	7.59	7.47	7.44	7.92	7.10	7.64
Index					+0.54	+0.42		-0.39	+0.53

Table 3-8
Bacterial Susceptibility to Carbenicillin

Microorganism	K-B (50µg) Zone Diameter (mm)	Tube Dilution MIC (µg/ml)	16 µg/ml				32 µg/ml			
			ATP	Index	Interpretation		ATP	Index	Interpretation	
			1 hr.	2 hr.	K-B	ATP Index	1 hr.	2 hr.	K-B	ATP Index
<i>E coli</i>	28	32	+1.05	+0.10	S	R → S	+0.95	-0.41	S	R → S
<i>Klebsiella</i>	0	>256	+0.97	+1.02	?	R	+1.08	+1.01	?	R
<i>Enterbacter</i>	20	128	+1.38	+0.87	?	R	+1.35	+0.76	?	R
<i>Proteus*</i>	30	2	+0.89	+0.33	S	R → S	+0.43	+0.07	S	R → S
			128 µg/ml				256 µg/ml			
<i>Pseudomonas</i>	19	128	+0.83	+0.70	S	R	+0.94	+0.64	S	R
<i>S aureus</i>	32	1	-0.88	-0.44	?	S	-1.13	-0.59	?	S
<i>S epidermidis</i>	27	4	-0.50	-0.73	?	S	-0.36	-0.48	?	S
<i>Enterococcus</i>	18.5	128	+0.98	-1.05	?	R	+1.00	+1.05	?	R

*In duplicate.

?There are no established K-B zone criteria for those species.

Table 3-9
Bacterial Susceptibility to Carbenicillin
at 16 µg/ml (B) and 32 µg/ml (C)

	A ₀	B ₀	C ₀	A ₁	B ₁	C ₁	A ₂	B ₂	C ₂
<i>E. coli</i> ATP (fg/ml)	9.95×10^6	9.60×10^6	9.35×10^6	4.06×10^7	4.37×10^7	3.80×10^7	2.23×10^8	1.38×10^7	2.76×10^6
bacteria/ml	3.62×10^7	3.49×10^7	3.40×10^7	1.47×10^8	1.59×10^8	1.38×10^8	8.11×10^8	5.00×10^7	1.00×10^7
log bact/ml	7.56	7.54	7.53	8.17	8.20	8.14	8.91	7.70	7.00
Index					+1.05	+0.95		+0.10	-0.41
<i>Klebsiella</i> ATP (fg/ml)	1.85×10^7	2.20×10^7	2.26×10^7	7.35×10^7	7.15×10^7	8.35×10^7	1.95×10^8	2.05×10^8	1.94×10^8
bacteria/ml	6.71×10^7	7.98×10^7	8.20×10^7	2.67×10^8	2.60×10^8	3.04×10^8	7.09×10^8	7.44×10^8	7.22×10^8
log bact/ml	7.83	7.90	7.91	8.43	8.41	8.48	8.85	8.87	8.86
Index					+0.97	+1.08		+1.02	+1.01
<i>Enterobacter</i> ATP (fg/ml)	1.04×10^7	9.50×10^6	1.10×10^7	1.90×10^7	2.40×10^7	2.35×10^7	8.15×10^7	6.15×10^7	4.99×10^7
bacteria/ml	3.76×10^7	3.45×10^7	3.98×10^7	6.89×10^7	8.73×10^7	8.55×10^7	2.96×10^8	2.24×10^8	1.81×10^8
log bact/ml	7.58	7.54	7.60	7.84	7.94	7.93	8.47	8.35	8.26
Index					+1.38	+1.35		+0.87	+0.76
<i>P. mirabilis</i> ATP (fg/ml)	8.05×10^6	8.80×10^6	9.90×10^6	3.06×10^7	2.32×10^7	1.44×10^7	1.55×10^8	3.50×10^6	1.49×10^7
bacteria/ml	2.93×10^7	3.20×10^7	3.27×10^7	1.11×10^8	8.42×10^7	5.22×10^7	5.62×10^8	1.27×10^6	5.42×10^7
log bact/ml	7.47	7.51	7.51	8.05	7.93	7.72	8.75	7.10	7.73
Index					+0.79	+0.43		-0.29	+0.20
<i>P. mirabilis</i> ATP (fg/ml)	6.15×10^6	6.35×10^6	6.30×10^6	2.35×10^7	1.88×10^7	1.08×10^7	1.14×10^8	2.06×10^6	5.25×10^6
bacteria/ml	2.24×10^7	2.31×10^7	2.29×10^7	8.55×10^7	6.83×10^7	3.94×10^7	4.14×10^8	7.50×10^6	1.91×10^7
log bact/ml	7.35	7.36	7.36	7.93	7.83	7.60	8.62	6.88	7.28
Index					+0.83	+0.43		-0.37	-0.06
<i>Pseudomonas</i> * ATP (fg/ml)	6.70×10^6	6.90×10^6	6.15×10^6	1.56×10^7	1.36×10^7	1.49×10^7	6.30×10^7	3.21×10^7	2.79×10^7
bact/ml	2.44×10^7	2.51×10^7	2.24×10^7	5.67×10^7	4.95×10^7	5.42×10^7	2.29×10^8	1.17×10^8	1.02×10^8
log bact/ml	7.39	7.40	7.35	7.75	7.69	7.73	8.36	8.07	8.01
Index					+0.83	+0.94		+0.70	+0.64
<i>S. aureus</i> ATP (fg/ml)	3.01×10^6	2.87×10^6	2.73×10^6	4.33×10^6	2.17×10^6	2.02×10^6	1.18×10^7	1.67×10^6	1.35×10^6
bact/ml	1.09×10^7	1.04×10^7	9.93×10^6	1.57×10^7	7.89×10^6	7.33×10^6	4.29×10^7	6.05×10^6	4.41×10^6
log bact/ml	7.04	7.02	7.00	7.20	6.90	6.86	7.63	6.78	6.69
Index					-0.58	-1.13		-0.44	-0.59
<i>S. epidermidis</i> ATP (fg/ml)	4.46×10^6	4.45×10^6	4.65×10^6	1.18×10^7	2.78×10^6	3.14×10^6	3.24×10^7	1.05×10^6	1.72×10^6
bact/ml	1.62×10^7	1.62×10^7	1.64×10^7	4.29×10^7	1.01×10^7	1.14×10^7	1.18×10^8	3.82×10^6	6.25×10^6
log bact/ml	7.21	7.21	7.23	7.63	7.00	7.06	8.07	6.58	6.80
Index					-0.50	-0.36		-0.73	-0.48
<i>Enterococcus</i> ATP (fg/ml)	8.85×10^6	9.80×10^6	9.45×10^6	2.25×10^7	2.19×10^7	2.22×10^7	7.25×10^7	8.20×10^7	8.15×10^7
bact/ml	3.22×10^7	3.56×10^7	3.44×10^7	8.18×10^7	7.96×10^7	8.05×10^7	2.64×10^8	2.98×10^8	2.96×10^8
log bact/ml	7.51	7.55	7.54	7.91	7.90	7.91	8.42	8.47	8.47
Index					+0.98	+1.00		+1.05	+1.05

* B = 128 µg/ml, C = 256 g/ml.

Table 3-10
P. aeruginosa Susceptibility to Carbenicillin
 at 128 µg/ml (B) with and without TX

Specimen	A ₀	B ₀	A ₁	B ₁	A ₂	B ₂	A ₃	B ₃	A ₄	B ₄	A ₅	B ₅	A ₆	B ₆	A ₂₄	B ₂₄
Dilution Read	3		3	3	4	3	4	2	5	2	5	2	5	7	7	1
Plate (1)	33		32	24	15	7	54	76	9	20	23	9	20	12	18	7
(2)	31		37	27	13	6	63	69	12	30	13	5	28	14	26	6
(3)	34		41	29	12	9	52	69	11	20	18	6	21	12	19	10
(4)	33		37	24	13	10	62	75	11	23	22	9	24	15	26	10
Total Colonies	131		147	104	53	32	231	289	43	93	76	29	93	53	89	33
Viable col/ml	1.31 × 10 ⁶		1.47 × 10 ⁶	1.04 × 10 ⁶	5.30 × 10 ⁶	3.20 × 10 ⁵	2.31 × 10 ⁷	2.59 × 10 ⁵	4.30 × 10 ⁷	9.30 × 10 ⁴	7.60 × 10 ⁷	9.90 × 10 ³	9.30 × 10 ⁷	5.30 × 10 ³	8.90 × 10 ⁹	3.30 × 10 ³
log viable col/ml	6.12		6.17	6.02	6.72	5.51	7.36	5.46	7.63	4.97	7.88	4.46	7.97	3.72	9.95	3.52
<u>With Tx</u>																
ATP (fg/ml)	4.08 × 10 ⁶	3.67 × 10 ⁶	8.80 × 10 ⁶	7.75 × 10 ⁶	2.65 × 10 ⁷	1.99 × 10 ⁷	9.40 × 10 ⁷	1.71 × 10 ⁷	1.25 × 10 ⁸	1.74 × 10 ⁷	1.40 × 10 ⁸	1.50 × 10 ⁷	1.92 × 10 ⁸	1.48 × 10 ⁷	1.72 × 10 ⁹	1.43 × 10 ⁶
bacteria/ml	1.48 × 10 ⁷	1.33 × 10 ⁷	3.20 × 10 ⁷	2.82 × 10 ⁷	2.64 × 10 ⁷	7.22 × 10 ⁷	3.42 × 10 ⁸	6.22 × 10 ⁷	4.53 × 10 ⁸	6.33 × 10 ⁷	5.09 × 10 ⁸	5.45 × 10 ⁷	6.98 × 10 ⁸	5.38 × 10 ⁷	6.24 × 10 ⁹	5.20 × 10 ⁶
log bact/ml	7.17	7.13	7.51	7.45	7.98	7.86	8.53	7.79	8.66	7.80	8.71	7.74	8.84	7.73	9.79	6.72
Index				+0.82		+0.85		+0.46		+0.42		+0.37		+0.34		-0.17
<u>Without Tx</u>																
ATP (fg/ml)	4.51 × 10 ⁶	3.94 × 10 ⁶	9.60 × 10 ⁶	9.35 × 10 ⁶	2.37 × 10 ⁷	2.63 × 10 ⁷	9.10 × 10 ⁷	4.00 × 10 ⁷	1.24 × 10 ⁸	6.00 × 10 ⁷	1.32 × 10 ⁸	3.79 × 10 ⁷	2.23 × 10 ⁸	3.00 × 10 ⁷	1.22 × 10 ⁹	1.78 × 10 ⁶
bacteria/ml	1.64 × 10 ⁷	1.43 × 10 ⁷	3.49 × 10 ⁷	3.40 × 10 ⁷	8.62 × 10 ⁷	9.55 × 10 ⁷	3.31 × 10 ⁸	1.45 × 10 ⁸	4.67 × 10 ⁸	2.18 × 10 ⁸	4.80 × 10 ⁸	1.38 × 10 ⁸	8.11 × 10 ⁸	1.09 × 10 ⁸	4.42 × 10 ⁹	6.45 × 10 ⁶
log bact/ml	7.21	7.16	7.54	7.53	7.94	7.98	8.52	8.16	8.67	8.34	8.68	8.14	8.91	8.04	9.65	6.81
Index				+0.97		+1.05		+0.73		+0.77		+0.63		+0.49		-0.16

Table 3-11
Bacterial Susceptibility to Cephalothin

Microorganism	K-B Zone Diameter (mm)	Tube Dilution MIC ($\mu\text{g/ml}$)	10 $\mu\text{g/ml}$				32 $\mu\text{g/ml}$			
			ATP	Index	Interpretation		ATP	Index	Interpretation	
			1 hr.	2 hr.	K-B	ATP Index	1 hr.	2 hr.	K-B	ATP Index
<i>E. coli</i>	19	32	+1.00	-0.02	S	R $\rightarrow$ S	-0.02	-0.68	S	S
<i>Klebsiella</i>	21	16	+0.94	-0.32	S	R $\rightarrow$ S	-0.23	-0.61	S	S
<i>Enterobacter</i>	0	>128	+0.98	+0.98	R	R	+1.02	+0.87	R	R
<i>P. mirabilis</i>	20	4	-0.25	+0.27	S	S $\rightarrow$ R	-0.29	+0.07	S	S
<i>Pseudomonas</i>	0	>128	+1.29	+1.18	R	R	+1.23	+1.15	R	R
<i>S. aureus</i>	32.5	<0.125	-1.16	-0.46	S	S	-1.00	-0.56	S	S
<i>S. epidermidis</i>	35	0.25	-0.46	-0.42	S	S	-0.59	-0.53	S	S
<i>Enterococcus</i>	14	32	+0.92	+0.87	R	R	+0.20	+0.07	R	I $\rightarrow$ S

Table 3-12
Bacterial Susceptibility to Tetracycline

Microorganism	K-B Zone Diameter (mm)	Tube Dilution MIC (μg/ml)	4 μg/ml				16 μg/ml			
			ATP	Index	Interpretation		ATP	Index	Interpretation	
			1 hr.	2 hr.	K-B	ATP Index	1 hr.	2 hr.	K-B	ATP Index
<i>E coli</i>	20	4	+0.11	+0.11	S	S	-0.22	-0.28	S	S
<i>Klebsiella</i>	19	8	-0.03	-0.00	S	S	-0.29	-0.28	S	S
<i>Enterobacter</i>	19	8	+0.02	+0.02	S	S	+0.03	-0.11	S	S
<i>P mirabilis</i>	0	128	+0.88	+0.90	R	R	-0.63	+0.15	R	S
<i>Pseudomonas</i>	0	64	+0.82	+0.18	R	R → (I)	-0.35	+0.14	R	S
<i>S aureus</i>	25.5	0.5	-1.08	-0.08	S	S	-1.32	-0.19	S	S
<i>S epidermidis</i>	0	>128	+1.28	+0.94	R	R	+1.28	+0.96	R	R
<i>Enterococcus</i>	20	1.0	-0.36	-0.14	S	S	-0.10	0.00	S	S

Table 3-13
Bacterial Susceptibility to Erythromycin

Microorganism	K-B (15 µg) Zone Diameter (mm)	Tube Dilution MIC (µg/ml)	2 µg/ml				8 µg/ml			
			ATP	Index	Interpretation		ATP	Index	Interpretation	
			1 hr.	2 hr.	K-B	ATP Index	1 hr.	2 hr.	K-B	ATP Index
<i>E. coli</i>	10.5	64	+0.95	+0.93	R	R	+0.91	+0.86	R	R
<i>Klebsiella</i>	11.0	128	+1.02	+1.00	R	R	+0.97	+0.88	R	R
<i>Enterobacter</i>	0	>128	+0.93	+0.95	R	R	+0.78	+0.91	R	R
<i>P. mirabilis</i>	0	>128	+1.03	+0.99	R	R	+1.14	+1.00	R	R
<i>Pseudomonas</i>	0	>128	+1.03	+0.94	R	R	+0.83	+0.74	R	R
<i>S. aureus</i>	28	0.25	0.00	+0.03	S	S	+0.04	0.00	S	S
<i>S. epidermidis</i>	29.5	0.25	+0.05	+0.01	S	S	-0.05	-0.04	S	S
<i>Enterococcus</i>	26	<0.125	0.00	-0.12	S	S	+0.06	-0.10	S	S

Table 3-14
Bacterial Susceptibility to Erythromycin
at 2 µg/ml (B) and 8 µg/ml (C)

	A ₀	B ₀	C ₀	A ₁	B ₁	C ₁	A ₂	B ₂	C ₂
<i>E. coli</i> ATP (fg/ml)	1.25 × 10 ⁷	1.48 × 10 ⁷	1.57 × 10 ⁷	4.45 × 10 ⁷	4.20 × 10 ⁷	3.96 × 10 ⁷	1.48 × 10 ⁸	1.24 × 10 ⁸	1.06 × 10 ⁸
bacteria/ml	4.53 × 10 ⁷	5.38 × 10 ⁷	5.69 × 10 ⁷	1.62 × 10 ⁸	1.53 × 10 ⁸	1.44 × 10 ⁸	5.38 × 10 ⁸	4.49 × 10 ⁸	3.84 × 10 ⁸
log bacteria/ml	7.66	7.73	7.76	8.21	8.18	8.16	8.73	8.65	8.58
Index					+0.95	+0.91		+0.93	+0.86
<i>Klebsiella</i> ATP (fg/ml)	1.78 × 10 ⁷	1.88 × 10 ⁷	1.73 × 10 ⁷	7.50 × 10 ⁷	7.70 × 10 ⁷	7.20 × 10 ⁷	1.84 × 10 ⁸	1.85 × 10 ⁸	1.41 × 10 ⁸
bacteria/ml	6.47 × 10 ⁷	6.84 × 10 ⁷	6.27 × 10 ⁷	2.73 × 10 ⁸	2.80 × 10 ⁸	2.62 × 10 ⁸	6.69 × 10 ⁸	6.71 × 10 ⁸	5.13 × 10 ⁸
log bact/ml	7.81	7.83	7.80	8.44	8.45	8.42	8.83	8.83	8.71
Index					+1.02	+0.97		+1.00	+0.88
<i>Enterobacter</i> ATP (fg/ml)	9.85 × 10 ⁶	9.25 × 10 ⁶	8.85 × 10 ⁶	2.64 × 10 ⁷	2.44 × 10 ⁷	2.10 × 10 ⁷	8.15 × 10 ⁷	7.25 × 10 ⁷	6.80 × 10 ⁷
bacteria/ml	3.58 × 10 ⁷	3.36 × 10 ⁷	3.22 × 10 ⁷	9.58 × 10 ⁷	8.85 × 10 ⁷	7.62 × 10 ⁷	2.96 × 10 ⁸	2.64 × 10 ⁸	2.47 × 10 ⁸
log bact/ml	7.55	7.53	7.51	7.98	7.95	7.88	8.47	8.42	8.39
Index					+0.93	+0.78		+0.95	+0.91
<i>P. mirabilis</i> ATP (fg/ml)	1.34 × 10 ⁷	1.38 × 10 ⁷	1.52 × 10 ⁷	6.30 × 10 ⁷	6.55 × 10 ⁷	7.95 × 10 ⁷	2.32 × 10 ⁸	2.31 × 10 ⁸	2.36 × 10 ⁸
bacteria/ml	4.85 × 10 ⁷	5.00 × 10 ⁷	5.53 × 10 ⁷	2.29 × 10 ⁸	2.38 × 10 ⁸	2.89 × 10 ⁸	8.44 × 10 ⁸	8.38 × 10 ⁸	8.58 × 10 ⁸
log bact/ml	7.67	7.70	7.74	8.36	8.38	8.46	8.93	8.92	8.93
Index					+1.03	+1.14		+0.99	+1.00
<i>Pseudomonas</i> ATP (fg/ml)	1.01 × 10 ⁷	1.06 × 10 ⁷	9.70 × 10 ⁶	2.53 × 10 ⁷	2.59 × 10 ⁷	2.14 × 10 ⁷	7.30 × 10 ⁷	6.50 × 10 ⁷	4.33 × 10 ⁷
bacteria/ml	3.67 × 10 ⁷	3.84 × 10 ⁷	3.53 × 10 ⁷	9.20 × 10 ⁷	9.40 × 10 ⁷	7.76 × 10 ⁷	2.65 × 10 ⁸	2.36 × 10 ⁸	1.57 × 10 ⁸
log bact/ml	7.56	7.58	7.55	7.96	7.97	7.89	8.42	8.37	8.20
Index					+1.03	+0.83		+0.94	+0.74
<i>S. aureus</i> ATP (fg/ml)	1.68 × 10 ⁶	1.77 × 10 ⁶	1.98 × 10 ⁶	3.43 × 10 ⁶	1.81 × 10 ⁶	1.87 × 10 ⁶	7.50 × 10 ⁶	1.90 × 10 ⁶	1.82 × 10 ⁶
bacteria/ml	6.11 × 10 ⁶	6.44 × 10 ⁶	7.20 × 10 ⁶	1.25 × 10 ⁷	6.58 × 10 ⁶	6.78 × 10 ⁶	2.73 × 10 ⁷	6.89 × 10 ⁶	6.62 × 10 ⁶
log bact/ml	6.79	6.81	6.86	7.10	6.82	6.83	7.44	6.84	6.82
Index					0.00	+0.04		+0.03	0.00
<i>S. epidermidis</i> ATP (fg/ml)	3.42 × 10 ⁶	2.51 × 10 ⁶	2.38 × 10 ⁶	7.60 × 10 ⁶	2.90 × 10 ⁶	2.65 × 10 ⁶	1.34 × 10 ⁷	2.80 × 10 ⁶	2.55 × 10 ⁶
bacteria/ml	1.24 × 10 ⁷	9.13 × 10 ⁶	8.64 × 10 ⁶	2.76 × 10 ⁷	1.05 × 10 ⁷	9.64 × 10 ⁶	4.87 × 10 ⁷	1.02 × 10 ⁷	9.27 × 10 ⁶
log bact/ml	7.09	6.96	6.94	7.44	7.02	6.98	7.69	7.01	6.97
Index					+0.05	-0.05		+0.01	-0.04
<i>Enterococcus</i> ATP (fg/ml)	7.55 × 10 ⁶	1.03 × 10 ⁷	9.45 × 10 ⁶	2.05 × 10 ⁷	9.20 × 10 ⁶	9.45 × 10 ⁶	1.14 × 10 ⁸	6.30 × 10 ⁶	7.10 × 10 ⁶
bacteria/ml	2.75 × 10 ⁷	3.73 × 10 ⁷	3.44 × 10 ⁷	7.44 × 10 ⁷	3.35 × 10 ⁷	3.44 × 10 ⁷	4.15 × 10 ⁸	2.44 × 10 ⁷	2.58 × 10 ⁷
log bact/ml	7.44	7.57	7.54	7.87	7.52	7.54	8.62	7.39	7.41
Index					0.00	+0.06		-0.12	-0.10

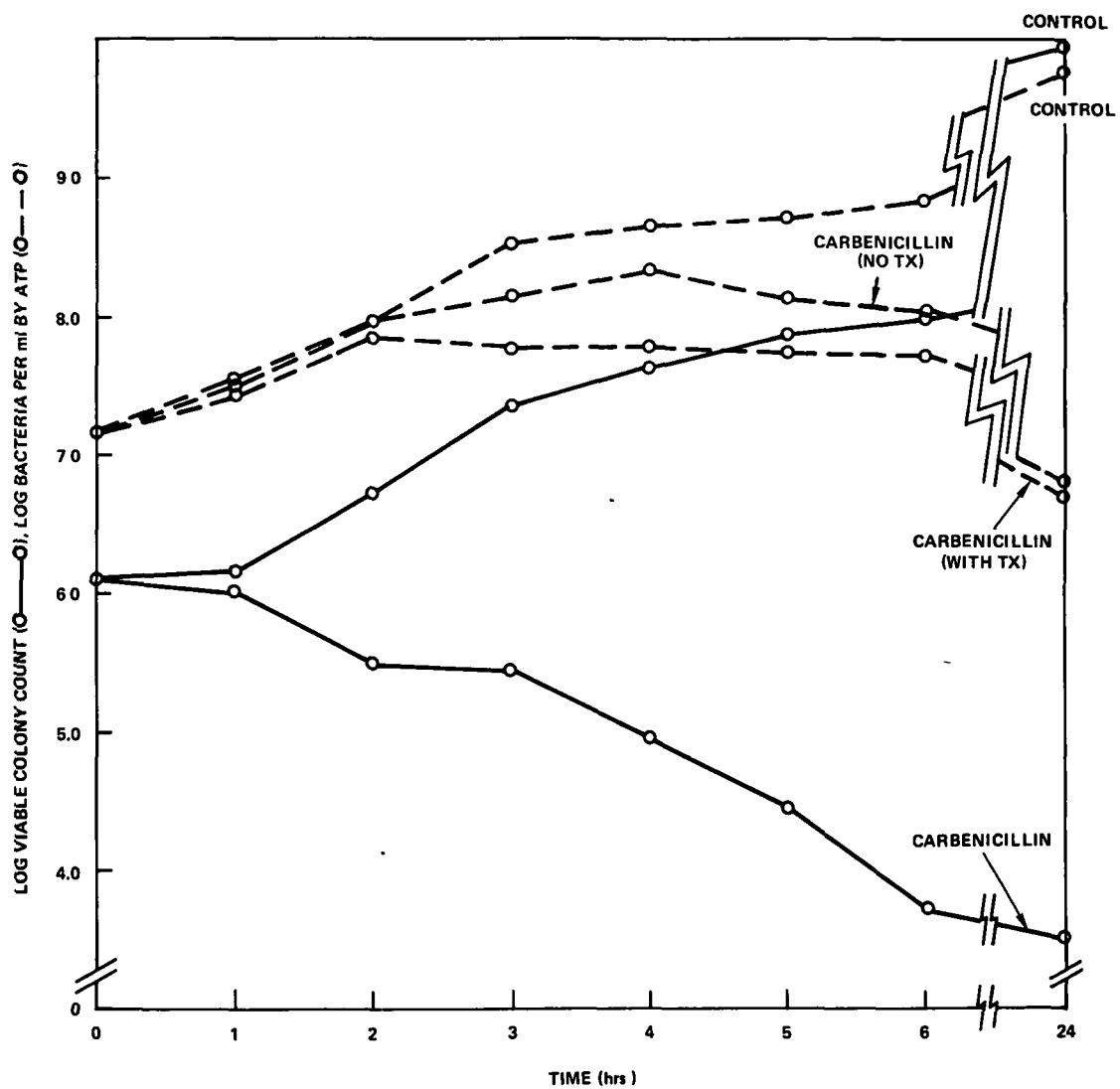


Figure 3-3. Time to lysis *Pseudomonas Aeruginosa* grown in the presence of carbenicillin (128 µg/ml) with and without TX.

Clindamycin

The distribution of sensitivities to clindamycin (tables 3-15 and 3-16) is clearly bimodal and susceptibility discrimination is relatively clear-cut. The results with an *enterococcus* are interesting in that although this organism was considered resistant to clindamycin by the agar diffusion technique, the MIC value of 8 $\mu\text{g/ml}$ suggests a more intermediate susceptibility that corresponds better with the ATP index. In this instance the apparent discrepancy is not serious.

Gentamicin

There is 100 percent correlation at both antibiotic concentrations of gentamicin (see table 3-17).

Sulfisoxazole

Preliminary results with sulfisoxazole (see tables 3-18, 3-19, 3-20 and 3-21) suggest several difficult problems.

Because bacteria undergo several generations until the effect of the sulfonamide become apparent (see table 3-21 and figure 3-4), very low inocula ($\sim 5 \times 10^2$ to 5×10^3 organisms/ml) must be used. There does not appear to be any consistent sulfonamide effect produced after 3 hours of log phase growth using an inoculum of $\sim 5 \times 10^4$ organisms/ml (see table 3-19, 3-20 and figure 3-5), making "rapid" sensitivity testing to this agent impossible. There will be further study of the kinetics of sulfonamide effect on bacterial cultures but it is doubtful that the methods and criteria used for antibiotics can be applied to sulfonamides.

SUMMARY

By careful selection of antibiotic concentration and duration of growth in the presence of the drug, we can exceed 90 percent correlation with the Kirby-Bauer agar diffusion technique.

The exceptions to the above generalization can be explained either by prolonged time to lysis with some β -lactam antibiotics or by prolonged delay in antibacterial effect as exemplified by the sulfonamides.

At times, the ATP index correlates better with the tube-dilution MIC than the Kirby-Bauer technique and apparent discrepancies in these instances are not serious.

The tentative criteria that we have adopted for the interpretation of the ATP index of inhibition may be subject to some revision once additional experience has been gained with this technique.

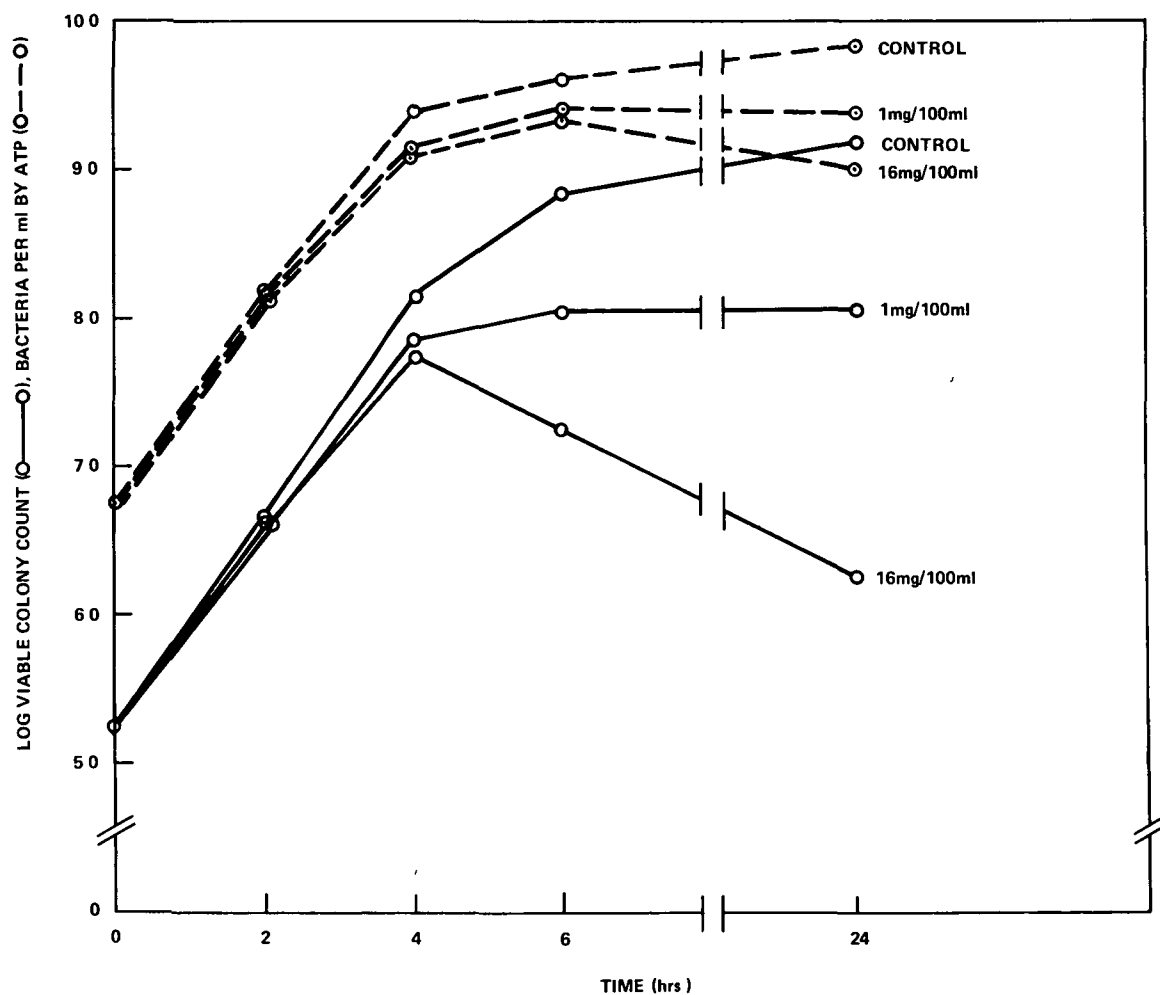


Figure 3-4. Change in bacterial ATP and colony counts with time when $\sim 10^3$ *E. coli* are grown in the presence of sulfisoxazole.

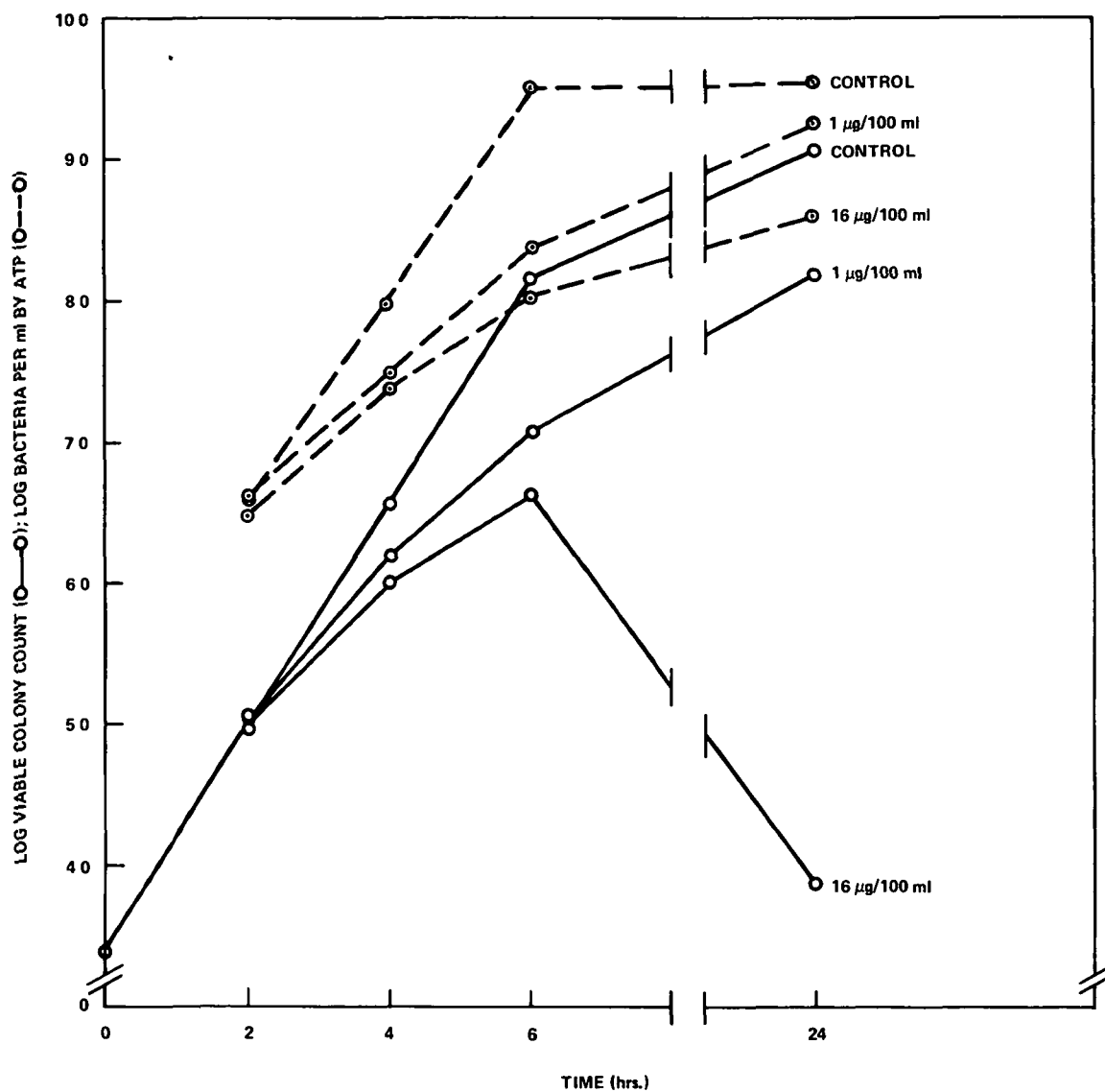


Figure 3-5. Change in bacterial ATP and colony counts with time when $\sim 10^5$ *E. coli* are grown in the presence of sulfisoxazole.

Table 3-15
Bacterial Susceptibility to Clindamycin

Microorganism	K-B (2 µg) Zone Diameter (mm)		Tube Dilution MIC (µg/ml)	2 µg/ml				8 µg/ml			
				ATP	Index	Interpretation		ATP	Index	Interpretation	
				1 hr.	2 hr.	K-B	ATP Index	1 hr.	2 hr.	K-B	ATP Index
<i>E. coli</i>	0	R	128	+0.97	+0.99	R	R	+0.97	+0.97	R	R
<i>Klebsiella</i>	0	R	>128	+1.12	+0.96	R	R	+1.04	+0.85	R	R
<i>Enterobacter</i>	0	R	>128	+1.26	+0.99	R	R	+0.79	+0.96	R	R
<i>P. mirabilis</i>	0	R	>128	+0.88	+1.04	R	R	+1.00	0.94	R	R
<i>Pseudomonas</i>	0	R	>128	+1.11	+1.32	R	R	+1.09	+1.18	R	R
<i>S. aureus</i>	26	S	<0.125	-0.42	-0.41	S	S	-0.58	-0.16	S	S
<i>S. epidermidis</i>	29	S	<0.125	-0.13	-0.07	S	S	-0.13	-0.15	S	S
<i>Enterococcus</i>	0	R	8	+0.19	+0.20	R	I	+0.28	+0.07	R	I → S

Table 3-16
Bacterial Susceptibility to Clindamycin
at 2 µg/ml (B) and 8 µg/ml (C)

	A ₀	B ₀	C ₀	A ₁	B ₁	C ₁	A ₂	B ₂	C ₂
<i>S. aureus</i> ATP (fg/ml)	2.15 × 10 ⁷	1.60 × 10 ⁷	1.40 × 10 ⁷	3.74 × 10 ⁷	1.71 × 10 ⁷	1.56 × 10 ⁷	8.15 × 10 ⁷	1.22 × 10 ⁷	1.73 × 10 ⁷
bacteria/ml	7.76 × 10 ⁷	5.79 × 10 ⁷	5.05 × 10 ⁷	1.35 × 10 ⁸	6.19 × 10 ⁷	5.65 × 10 ⁷	2.95 × 10 ⁸	4.42 × 10 ⁷	6.24 × 10 ⁷
log bacteria/ml	7.89	7.76	7.70	8.13	7.79	7.75	8.47	7.65	7.80
Index					-0.42	-0.58		-0.41	-0.15
<i>S. epidermidis</i> ATP (fg/ml)	3.29 × 10 ⁶	2.54 × 10 ⁶	2.35 × 10 ⁶	9.45 × 10 ⁶	2.92 × 10 ⁶	2.90 × 10 ⁶	1.58 × 10 ⁷	2.94 × 10 ⁶	2.61 × 10 ⁶
bacteria/ml	1.19 × 10 ⁷	9.19 × 10 ⁶	8.51 × 10 ⁶	3.42 × 10 ⁷	1.06 × 10 ⁷	1.05 × 10 ⁷	5.70 × 10 ⁷	1.06 × 10 ⁷	9.45 × 10 ⁶
log bacteria/ml	7.08	6.96	6.93	7.53	7.02	7.02	7.76	7.03	6.98
Index					-0.13			-0.07	-0.15
<i>Enterococcus</i> ATP (fg/ml)	1.13 × 10 ⁷	1.62 × 10 ⁷	1.69 × 10 ⁷	7.35 × 10 ⁷	1.58 × 10 ⁷	1.92 × 10 ⁷	2.52 × 10 ⁸	1.21 × 10 ⁷	1.42 × 10 ⁷
bacteria/ml	4.07 × 10 ⁷	5.86 × 10 ⁷	6.12 × 10 ⁷	2.66 × 10 ⁸	5.72 × 10 ⁷	6.95 × 10 ⁷	9.10 × 10 ⁸	4.36 × 10 ⁷	5.14 × 10 ⁷
log bacteria/ml	7.61	7.77	7.79	8.42	7.76	7.84	8.96	7.64	9.71
Index					+0.19	+0.28		+0.2	+0.07
<i>E. coli</i> ATP (fg/ml)	1.27 × 10 ⁷	1.23 × 10 ⁷	1.35 × 10 ⁷	7.00 × 10 ⁷	6.80 × 10 ⁷	6.85 × 10 ⁷	2.15 × 10 ⁸	1.90 × 10 ⁸	1.98 × 10 ⁸
bacteria/ml	4.60 × 10 ⁷	4.49 × 10 ⁷	4.91 × 10 ⁷	2.55 × 10 ⁸	2.47 × 10 ⁸	2.49 × 10 ⁸	7.78 × 10 ⁸	7.15 × 10 ⁸	7.15 × 10 ⁸
log bacteria/ml	7.66	7.65	7.69	8.41	8.39	8.40	8.89	8.85	8.85
Index					+0.97	+0.99		+0.97	+0.97
<i>Klebsiella</i> ATP (fg/ml)	2.41 × 10 ⁷	2.54 × 10 ⁷	2.41 × 10 ⁷	1.32 × 10 ⁸	1.64 × 10 ⁸	1.41 × 10 ⁸	3.75 × 10 ⁸	3.33 × 10 ⁸	2.45 × 10 ⁹
bacteria/ml	8.77 × 10 ⁷	9.22 × 10 ⁷	8.75 × 10 ⁷	4.80 × 10 ⁸	5.94 × 10 ⁸	5.09 × 10 ⁸	1.36 × 10 ⁹	1.21 × 10 ⁹	8.87 × 10 ⁸
log bacteria/ml	7.94	7.96	7.94	8.68	8.77	8.71	9.13	9.08	8.95
Index					+1.12	+1.04		+0.96	+0.85
<i>Enterobacter</i> ATP (fg. ml)	1.27 × 10 ⁷	1.30 × 10 ⁷	1.30 × 10 ⁷	4.83 × 10 ⁷	4.80 × 10 ⁷	3.66 × 10 ⁷	1.18 × 10 ⁸	1.11 × 10 ⁸	1.08 × 10 ⁸
bacteria/ml	4.61 × 10 ⁷	4.69 × 10 ⁷	4.69 × 10 ⁷	1.75 × 10 ⁸	1.74 × 10 ⁸	1.32 × 10 ⁸	4.25 × 10 ⁸	4.20 × 10 ⁸	3.91 × 10 ⁸
log bacteria/ml	7.66	7.67	7.67	8.24	8.24	8.12	8.63	8.62	8.59
Index					+1.26	+0.79		+0.99	+0.96
<i>P. mirabilis</i> ATP (fg/ml)	1.89 × 10 ⁷	2.05 × 10 ⁷	2.15 × 10 ⁷	8.25 × 10 ⁷	6.90 × 10 ⁷	8.30 × 10 ⁷	2.22 × 10 ⁸	2.47 × 10 ⁸	1.96 × 10 ⁸
bacteria/ml	6.82 × 10 ⁷	7.40 × 10 ⁷	7.76 × 10 ⁷	2.99 × 10 ⁸	2.50 × 10 ⁸	3.00 × 10 ⁸	8.04 × 10 ⁸	8.92 × 10 ⁸	1.08 × 10 ⁸
log bacteria/ml	7.83	7.87	7.89	8.48	8.40	8.48	8.91	8.95	8.85
Index					+0.88	+1.00		+1.04	+0.94
<i>Pseudomonas</i> ATP (fg/ml)	4.33 × 10 ⁶	4.47 × 10 ⁶	4.35 × 10 ⁶	1.20 × 10 ⁷	1.35 × 10 ⁷	1.31 × 10 ⁷	3.21 × 10 ⁷	6.10 × 10 ⁷	4.57 × 10 ⁷
bacteria/ml	1.51 × 10 ⁷	1.61 × 10 ⁷	1.57 × 10 ⁷	4.34 × 10 ⁷	4.87 × 10 ⁷	4.71 × 10 ⁷	1.16 × 10 ⁸	2.21 × 10 ⁸	1.65 × 10 ⁸
log bacteria/ml	7.19	7.21	7.20	7.64	7.69	7.68	8.06	8.34	8.22
Index					+1.11	+1.09		+1.32	+1.18

Table 3-17
Bacterial Susceptibility to Gentamicin

Microorganism	K-B Zone Diameter (mm)	Tube Dilution MIC ($\mu\text{g/ml}$)	6 $\mu\text{g/ml}$				12 $\mu\text{g/ml}$			
			ATP	Index	Interpretation		ATP	Index	Interpretation	
			1 hr.	2 hr.	K-B	ATP Index	1 hr.	2 hr.	K-B	ATP Index
<i>E. coli</i>	22	4	-1.80	-1.53	S	S	-2.02	-1.68	S	S
<i>Klebsiella</i>	22	2	-1.89	-0.64	S	S	-1.86	-0.98	S	S
<i>Enterobacter</i>	24	2	-1.60	-1.10	S	S	-1.89	-1.03	S	S
<i>P. mirabilis</i>	22	8	-0.59	-0.41	S	S	-0.86	-0.41	S	S
<i>Pseudomonas</i>	20.5	2	-1.10	-0.53	S	S	-1.05	-0.49	S	S
<i>S. aureus</i>	24	0.25	-1.20	-0.37	S	S	-1.47	-0.41	S	S
<i>S. epidermidis</i>	30	0.125	-0.06	-0.01	S	S	-0.16	-0.03	S	S
<i>Enterococcus</i>	13	64	+1.08	+0.93	R	R	+1.10	+0.98	R	R

Table 3-18
Bacterial Susceptibility to Sulfisoxazole
at 8 µg/100 ml (B) and 32 µg/100 ml (C)

	A ₀	B ₀	C ₀	A ₁	B ₁	C ₁	A ₂	B ₂	C ₂
<i>E. coli</i> ATP (fg/ml)	1.98×10^7	2.36×10^7	2.12×10^7	6.90×10^7	7.10×10^7	7.20×10^7	2.25×10^8	2.09×10^8	1.94×10^8
bacteria/ml	7.17×10^7	8.53×10^7	7.67×10^7	2.50×10^8	2.51×10^8	2.61×10^8	8.15×10^8	7.55×10^8	7.00×10^8
log bacteria/ml	7.86	7.93	7.89	8.40	8.41	8.42	8.91	8.88	8.85
Index					+1.02	+1.04		+0.97	+0.94
<i>Klebsiella</i> ATP (fg. ml)	1.61×10^7	1.72×10^7	1.70×10^7	6.80×10^7	8.55×10^7	7.10×10^7	1.80×10^8	1.83×10^8	2.02×10^8
bacteria/ml	5.81×10^7	6.21×10^7	6.15×10^7	2.47×10^8	3.11×10^8	2.58×10^8	6.53×10^8	6.65×10^8	7.33×10^8
log bacteria/ml	7.76	7.79	7.79	8.39	8.49	8.41	8.81	8.82	8.86
Index					+1.16	+1.03		+1.01	+1.05
<i>Enterobacter</i> ATP (fg/ml)	1.20×10^7	1.04×10^7	1.06×10^7	3.27×10^7	3.00×10^7	2.55×10^7	8.45×10^7	9.30×10^7	8.20×10^7
bacteria/ml	4.35×10^7	3.78×10^7	3.85×10^7	1.19×10^8	1.09×10^8	9.27×10^7	3.07×10^8	3.38×10^8	2.98×10^8
log bacteria/ml	7.64	7.58	7.59	8.07	8.04	7.97	8.49	8.53	8.47
Index					+0.93	+0.77		+1.05	+0.98
<i>P. mirabilis</i> ATP (fg/ml)	1.72×10^7	1.66×10^7	1.46×10^7	7.95×10^7	8.55×10^7	8.15×10^7	3.32×10^8	1.89×10^8	2.46×10^8
bacteria/ml	6.24×10^7	6.04×10^7	5.31×10^7	2.89×10^8	3.11×10^8	2.96×10^8	1.21×10^9	6.85×10^8	8.93×10^8
log bacteria/ml	7.79	7.78	7.73	8.46	8.49	8.47	9.08	8.84	8.95
Index					+1.04	+1.01		+0.81	+0.90
<i>Pseudomonas</i> ATP (fg/ml)	7.25×10^6	7.50×10^6	8.80×10^6	1.85×10^7	1.94×10^7	1.94×10^7	4.95×10^7	6.35×10^7	6.10×10^7
bacteria/ml	2.64×10^7	2.73×10^7	3.20×10^7	6.71×10^7	7.05×10^7	7.05×10^7	1.80×10^8	2.31×10^8	2.22×10^8
log bacteria/ml	7.42	7.44	7.51	7.83	7.85	7.85	8.26	8.36	8.35
Index					+1.05	+1.05		+1.12	+1.11
<i>S. aureus</i> ATP (fg/ml)	3.38×10^6	3.18×10^6	3.07×10^6	8.65×10^6	9.15×10^6	9.05×10^6	4.25×10^7	3.95×10^7	4.47×10^7
bacteria/ml	1.23×10^7	1.15×10^7	1.11×10^7	3.15×10^7	3.33×10^7	3.29×10^7	1.55×10^8	1.43×10^8	1.63×10^8
log bacteria/ml	7.09	7.06	7.05	7.50	7.52	7.52	8.19	8.16	8.21
Index					+1.05	+1.05		+0.97	+1.02
<i>S. epidermidis</i> ATP (fg/ml)	1.50×10^6	1.59×10^6	1.24×10^6	1.73×10^6	1.85×10^6	1.89×10^6	3.98×10^6	3.06×10^6	3.75×10^6
bacteria/ml	5.44×10^6	5.76×10^6	4.51×10^6	6.27×10^6	6.73×10^6	6.85×10^6	1.45×10^7	1.11×10^7	1.36×10^7
log bacteria/ml	6.74	6.76	6.65	6.80	6.83	6.84	7.16	7.05	7.13
Index					+1.5	+1.67		+0.74	+0.93
<i>Enterococcus</i> ATP (fg/ml)	3.08×10^6	3.21×10^6	3.19×10^6	5.75×10^6	6.05×10^6	5.85×10^6	1.35×10^7	1.26×10^7	1.22×10^7
bacteria/ml	1.12×10^7	1.17×10^7	1.16×10^7	2.09×10^7	2.20×10^7	2.13×10^7	4.89×10^7	4.56×10^7	4.44×10^7
log bacteria/ml	7.05	7.07	7.06	7.32	7.34	7.33	7.69	7.66	7.65
Index					+1.07	+1.04		+0.95	+0.94

Table 3-19
Bacterial Susceptibility to Sulfisoxazole at 16 $\mu\text{g}/100\text{ ml}$

Inoculum $\sim 5 \times 10^4$ viable	A ₀	A ₃	B ₃
<i>E coli</i> ATP (fg/ml)	3.74×10^7	4.84×10^9	8.85×10^9
log fg/ml	7.57	9.68	9.45
Index			+0.91
<i>Klebsiella</i> ATP (fg/ml)	1.83×10^7	4.82×10^9	4.09×10^9
log fg/ml	7.26	9.68	9.61
Index			+0.97
<i>Enterobacter</i> ATP (fg/ml)	8.35×10^6	7.35×10^8	2.42×10^8
log fg/ml	6.92	8.87	8.38
Index			+0.75
<i>P mirabilis</i> ATP (fg/ml)	2.17×10^7	4.87×10^9	2.42×10^8
log fg/ml	7.34	9.69	8.38
Index			+0.44
<i>Pseudomonas</i> ATP (fg/ml)	7.45×10^6	7.95×10^8	1.95×10^8
log lg/ml	6.87	8.90	8.29
Index			+0.70
<i>S aureus</i> ATP (fg/ml)	1.77×10^7	1.92×10^9	1.73×10^9
log fg/ml	7.25	9.28	9.24
Index			+0.98
<i>S epidermidis</i> ATP (fg/ml)	1.36×10^7	5.75×10^8	6.05×10^8
log fg/ml	7.13	8.76	8.78
Index			+1.01
<i>Enterococcus</i> ATP (fg/ml)	1.80×10^7	4.58×10^8	4.47×10^8
log fg/ml	7.26	8.66	8.65
Index			+0.99

Table 3-20
Bacterial Susceptibility to Sulfisoxazole
at 1 µg/100 ml (B) and 16 µg/100 ml (C)

Specimen (Inoculum ~ 10 ⁵ /ml)	A ₀	B ₀	C ₀	A ₂	B ₂	C ₂	A ₄	B ₄	C ₄	A ₆	B ₆ *	C ₆ **	A ₂₄	B ₂₄
Dilution Read	2			4	4	4	5	5	5	6		4	6	5
Plate (1)	38			14	11	7	46	19	12	19		48	35	34
(2)	44			12	10	16	34	21	21	19		41	38	28
(3)	50			7	9	9	29	16	8	12		47	43	26
(4)	37			9	11	9	39	18	18	19		46	40	22
Total Colonies	169			42	41	41	148	74	59	69		182	156	110
Viable col/ml	1.69 x 10 ⁵			4.2 x 10 ⁶	4.1 x 10 ⁶	4.1 x 10 ⁶	1.48 x 10 ⁸	7.4 x 10 ⁷	5.9 x 10 ⁷	6.9 x 10 ⁸		1.82 x 10 ⁷	1.56 x 10 ⁹	1.10 x 10 ⁸
log viable col/ml	5.23			6.62	6.61	6.61	8.17	7.87	7.77	8.84		7.26	9.19	8.04
ATP (fg/ml)	1.55 x 10 ⁶	1.54 x 10 ⁶	1.57 x 10 ⁶	4.20 x 10 ⁷	3.91 x 10 ⁷	3.90 x 10 ⁷	6.80 x 10 ⁸	3.86 x 10 ⁸	3.68 x 10 ⁸	1.13 x 10 ⁹	7.05 x 10 ⁸	6.35 x 10 ⁸	1.84 x 10 ⁹	6.60 x 10 ⁸
bacteria/ml	5.61 x 10 ⁶	5.56 x 10 ⁶	5.66 x 10 ⁶	1.52 x 10 ⁸	1.42 x 10 ⁸	1.41 x 10 ⁸	2.46 x 10 ⁹	1.40 x 10 ⁹	1.33 x 10 ⁹	4.07 x 10 ⁹	2.55 x 10 ⁹	2.30 x 10 ⁹	6.64 x 10 ⁹	2.39 x 10 ⁹
log bact/ml	6.75	6.74	6.75	8.18	8.15	8.15	9.39	9.15	9.12	9.61	9.41	9.36	9.82	9.38
Index					+0.98	+0.98		+0.91	+0.90		+0.93	+0.91		+0.86

*Contaminated

**2 populations.

Table 3-21
Bacterial Susceptibility to Sulfisoxazole
at 1 $\mu\text{g/ml}$ (B) and 16 $\mu\text{g/ml}$ (C)

Specimen (Inoculum $\sim 10^3/\text{ml}$)	A ₀	B ₀	C ₀	A ₂	B ₂	C ₂	A ₄	B ₄	C ₄	A ₆	B ₆	C ₆	A ₂₄	B ₂₄
Dilution Read	1			2	2	2	4	3	3	5	4	4	6	5
Plate (1)	4			27	29	24	6	42	19	35	29	5	35	34
(2)	7			24	24	24	10	41	25	30	24	16	28	37
(3)	8			17	33	35	6	35	30	28	33	14	25	40
(4)	6			28	32	27	12	42	25	39	33	8	30	42
Total Colonies	25			96	118	110	34	160	99	142	119	43	118	153
Viable col/ml	2.5×10^3			9.6×10^4	1.18×10^5	1.10×10^5	3.4×10^6	1.60×10^6	9.9×10^5	1.42×10^8	1.19×10^7	4.3×10^6	1.18×10^9	1.53×10^8
log viable col/ml	3.40			4.98	5.07	5.04	6.53	6.20	6.05	8.15	7.08	6.63	9.07	8.18
ATP (fg/ml)	1.10×10^5	4.82×10^5	3.56×10^5	1.08×10^6	1.13×10^6	8.25×10^5	2.54×10^7	8.55×10^6	6.85×10^6	8.50×10^8	6.70×10^7	3.03×10^7	9.15×10^8	9.67×10^8
bacteria/ml	3.19×10^6	1.74×10^6	1.29×10^6	3.91×10^6	4.09×10^6	2.99×10^6	9.19×10^7	3.10×10^7	2.48×10^7	3.08×10^9	2.43×10^8	1.10×10^8	3.31×10^9	1.69×10^9
log bact/ml	6.50	6.24	6.11	6.59	6.61	6.48	7.96	7.49	7.39	9.49	8.38	8.04	9.52	9.23
Index					+1.22	-0.22		+0.68	+0.61		+0.63	+0.52		+0.90

SECTION 4

INTERIM PROGRESS REPORT, OCTOBER 1973: EFFECT OF ANTIMICROBIAL AGENTS ON BACTERIAL ATP

INTRODUCTION

The purpose of the preliminary experiments performed was to establish that the reduction of bacterial ATP content by antimicrobial agents is a valid measure of drug effect. This effort included the work necessary to optimize the luciferase assay for ATP for application to pure cultures in broth, establish the optimum duration of preincubation and incubation, determine for each antimicrobial agent the concentration which results in the best discrimination between sensitive and resistant strains, and to develop an index of inhibition to quantitate the drug effect on bacterial ATP.

The results of this aspect of the work are summarized in table 4-1, which compares the broth dilution MIC and agar disc-diffusion method with the ATP index.

The ATP index is expressed as the mean of duplicate determinations except in the one instance (*P. mirabilis* versus penicillin G at 8 $\mu\text{g/ml}$) where these were markedly different.

There is a 94 percent agreement in the results of the three methods. The discordant results will be analyzed in detail in the discussion of problems below. An ATP index of inhibition has been developed that, in a preliminary evaluation, has a 94-percent correlation with standard agar disc-diffusion sensitivity tests after only 3 hours.

PROJECTED STUDIES

The size of the bacterial inoculum is a critical variable in determining the results of standard microbial susceptibility tests. It is possible that this may not be as critical a determinant in rapid sensitivity tests. To evaluate this effect, the basic procedure is repeated using three bacterial inoculum sizes: 5×10^4 , 5×10^6 , and 5×10^7 colony-forming units/ml. For the lowest inoculum size (5×10^4), an alternative ATP assay, the short centrifugation procedure (Chappelle et al., 1975), has been developed to give the increased sensitivity required.

SOME PROBLEMS WITH RAPID ANTIMICROBIAL SUSCEPTIBILITY TESTING WITH SPECIAL REFERENCE TO THE ATP INDEX OF INHIBITION

Conventional susceptibility testing requiring overnight incubation reflects the final result of a dynamic and complex interaction between the antimicrobial agent and the microorganism. All currently described methods attempting to rapidly quantitate drug effect, whether by hemoglobin reduction (Sellers et al., 1969), sensitive turbidometric techniques, microcalorimetry (Binford et al., 1973), radiometric means (DeBlanc et al., 1972; DeLand, 1972; Isenberg et al., 1971; McKie et al., 1973; Thornsberry et al., 1973; McGowan et al., 1973), early zone measurement of the standard agar disc-diffusion technique (Boyle et al., 1973; Barry et al., 1973), or as we have done, measuring bacterial ATP content, will have many similar problems.

Four major factors will affect all of these tests, and they are as follows: the particular property of the bacterium measured, the mode of action of the antimicrobial agent, the mechanism of resistance to the antimicrobial agent, and the fact that a bacterial inoculum in the order of 10^6 colony-forming units/ml may behave in a heterogenous manner to an antimicrobial agent.

DETERMINATION OF THE PROPERTY OF THE BACTERIUM MEASURED BY ATP

The majority of the ATP of aerobic bacteria is supplied by oxidative phosphorylation, which in turn is coupled to bacterial respiratory chains. Thus, the bacterial ATP content is related to the functional integrity of these two processes and it declines if bacteria are exposed to uncouplers or are made anaerobic (Harold, 1972). Although none of the commonly used antimicrobial agents acts directly on these processes, it is evident that indirectly they may have profound consequences on bacterial ATP.

In addition to these general principles, several notable factors influence the bacterial ATP content. These are: the microbial species; the growth cycle (rapidly multiplying log phase cultures contain more ATP/bacterium than lag phase or early stationary phase cultures); the composition and redox state of the growth medium; and the method of extraction of ATP and its assay.

Although attempts have been made to correlate bacterial ATP with absolute numbers of microorganisms or colony-forming units (using a conversion factor of 2.75×10^{-1} fg ATP/bacterium), the above data suggest that this supposition is invalid. Changes in bacterial ATP more closely parallel the turbidity (or mass) of the culture and since 50 percent of the dry weight of *E. coli* is protein, it may be inferred that bacterial ATP closely parallels the bacterial protein content.

EFFECT OF MODE OF ACTION OF ANTIMICROBIAL DRUGS ON RAPID SENSITIVITY TESTING

One of the shortcomings of agar disc-diffusion sensitivity testing is that it gives no information about the mode of action of the particular agent. Our experience to date clearly demonstrates that the early changes in bacterial ATP content are dependent upon the mechanism of action of the drug.

Agents that Alter Protein Synthesis

In general terms, these agents exert their antibacterial effect by acting on bacterial ribosomes. We have found that gentamicin, tetracycline, chloramphenicol, erythromycin, and clindamycin rapidly inhibit bacterial ATP synthesis in susceptible species and that sensitivity or resistance to the agent can be determined within 1 hour. A "bacteriocidal" agent, such as gentamicin, produces a rapid fall in bacterial ATP, suggesting a profound effect on the functional integrity of the bacterium. A bacteriostatic agent such as tetracycline inhibits further ATP synthesis without any actual significant drop in bacterial ATP content (figure 4-1).

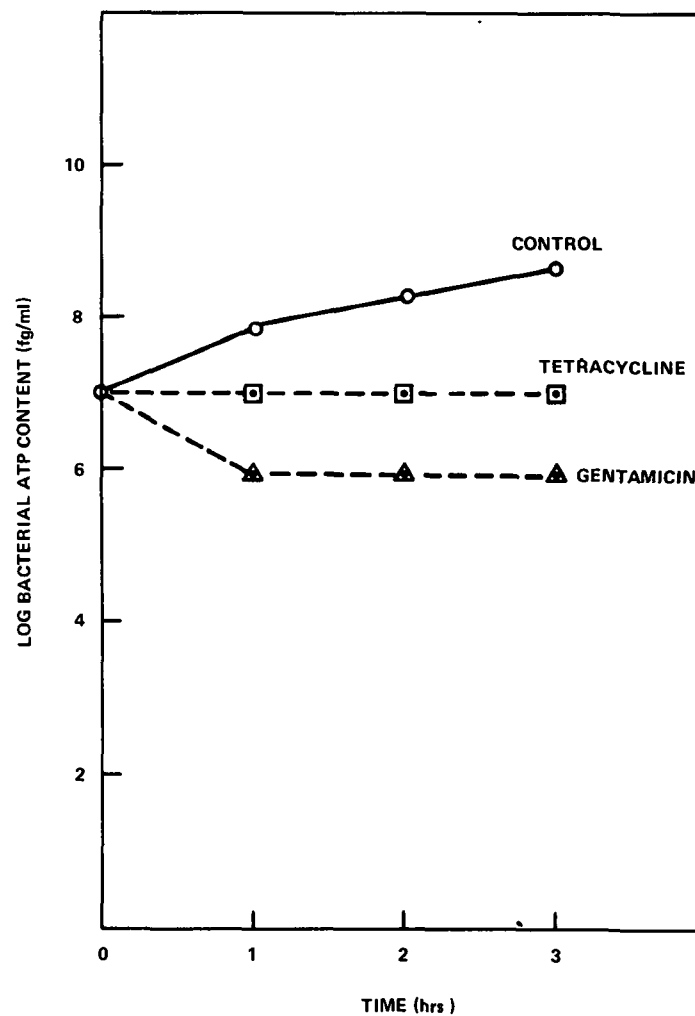
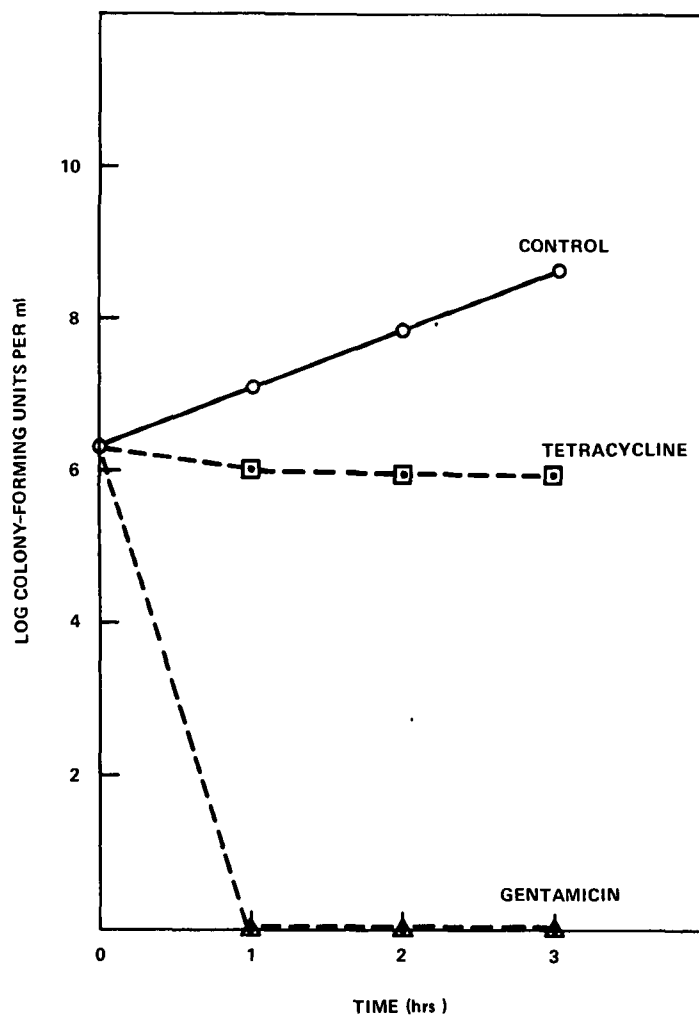


Figure 4-1. Growth inhibition of *Klebsiella aerogenes* 07220 by tetracycline at 8 $\mu\text{g/ml}$ ($\square$ — $\square$) and gentamicin at 2 $\mu\text{g/ml}$ (Δ — Δ); Comparison of viable counts and bacterial ATP content.

Cell Wall-Active (β -Lactam) Antibiotics

When a β -lactam antibiotic is added to a sensitive gram-negative bacterium in the logarithmic phase of growth, the bacterium undergoes a series of morphological changes from filamentous to bulbous or "bow tie" forms to spheroplasts before finally undergoing osmotic lysis (Greenwood and O'Grady, 1973). These changes are related to drug-induced alterations in the cell wall structure and may be dependent on the relative inhibitory effect of β -lactam antibiotics on two different enzymes, an endopeptidase concerned with cell division and a glycosidase concerned with cell growth (Hartmann et al., 1972). The rate at which these changes occur is dependent on the concentration of the drug, the type and rate of production of β -lactamase by the bacterium, and the stability of the drug to β -lactamase. In addition, the time to lysis is dependent on the osmolality of the medium (being more rapid at lower osmolalities) and the osmotic susceptibility of the species with damaged cell walls (*E. coli* being more susceptible than *P. mirabilis*). Continuous turbidometric recordings of these counts show increasing opacification of the broth culture (despite lack of cell division) for varying lengths of time after the addition of the drug until lysis occurs (Greenwood and O'Grady, 1973). Bacterial protein synthesis is not inhibited by this class of drugs.

When bacterial ATP content is used as a measure of β -lactam antibiotic effect on lag-phase cultures, the drop in ATP (indicating sensitivity) coincides with the time to achieve lysis. The time to achieve lysis has varied from less than 1 hour to greater than 6 hours. The prolonged time to achieve lysis seen with penicillin G and ampicillin against *P. mirabilis* (figure 4-2) and carbenicillin against *P. aeruginosa* (> 6 hours) limits the rapidity by which sensitivity can be determined by techniques that measure bacterial mass, protein synthesis, ATP, or other metabolic parameters apart from cell-wall synthesis.

In addition, there may be some delay in the expression of the organism's ability to produce sufficient quantities of a drug-inactivating enzyme (such as β -lactamase), the genetic capability of which implies resistance. This problem is particularly pertinent to penicillinase-producing *S. aureus*, which frequently appears sensitive to penicillin after only a few hours of growth (DeBlanc et al., 1972).

Sulfonamides

This class of chemotherapeutic agents act as competitive inhibitors of p-amino benzoic acid in the biosynthetic reaction to form dihydrofolate in bacteria. *In vitro* sensitivity testing to sulfonamides has always presented special difficulties due to the presence of substances in many microbiological media that inhibit the antibacterial action of sulfonamides (although they can be neutralized by the addition of lysed horse blood); and the necessity to use a very small inoculum of organisms, since bacteria may undergo many generations (Garrett and Wright, 1967), before any drug effect becomes apparent.

The latter point effectively precludes "rapid" measurement of drug effect; as would be expected, the ATP index consistently gives false resistant results (figure 4-3).

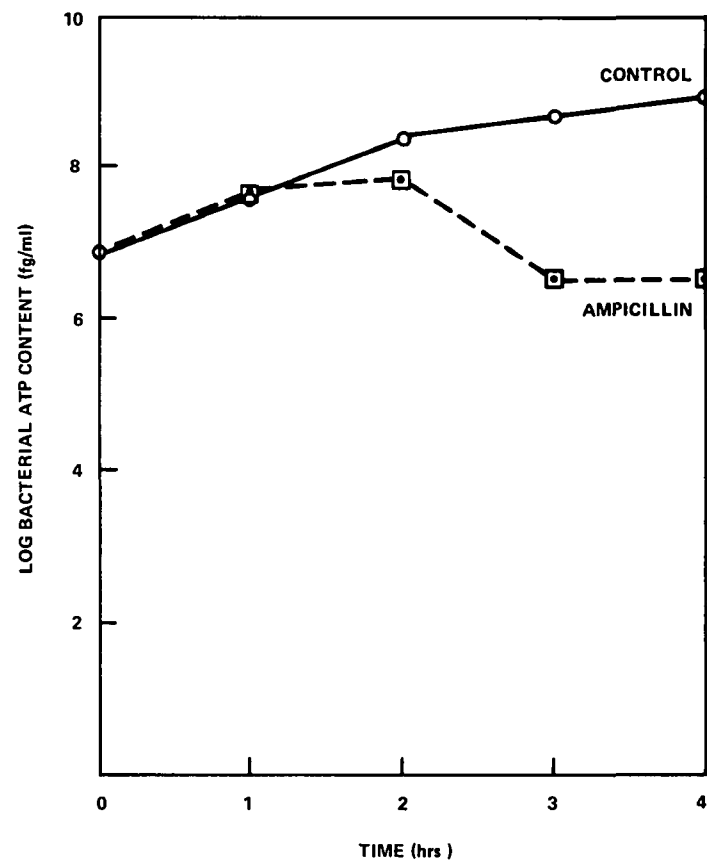
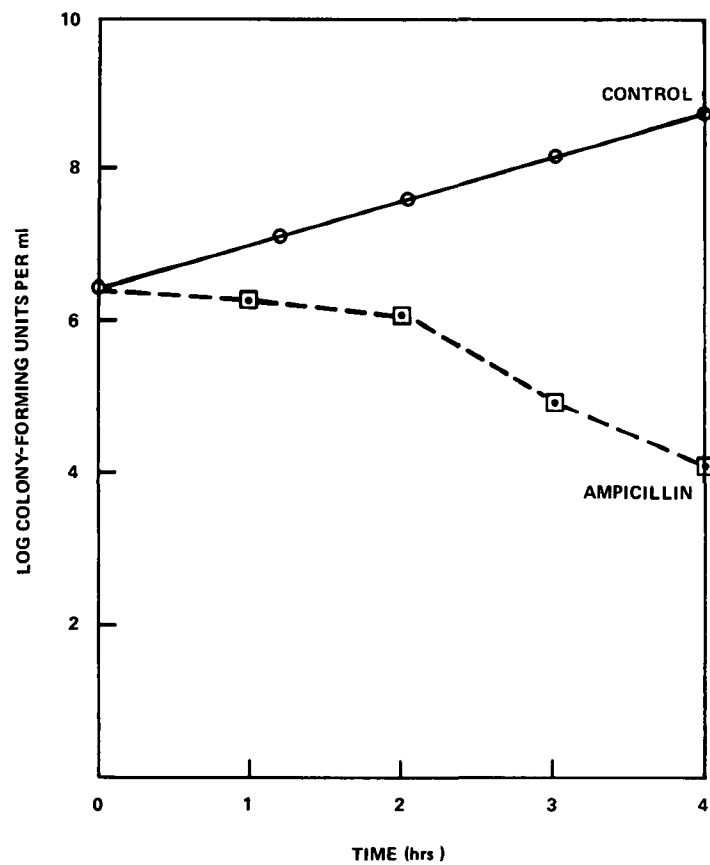


Figure 4-2. Growth inhibition of *P. mirabilis* 04583 by ampicillin at 1 μ g/ml ($\square$ — $\square$); Comparison of viable counts and bacterial ATP content.

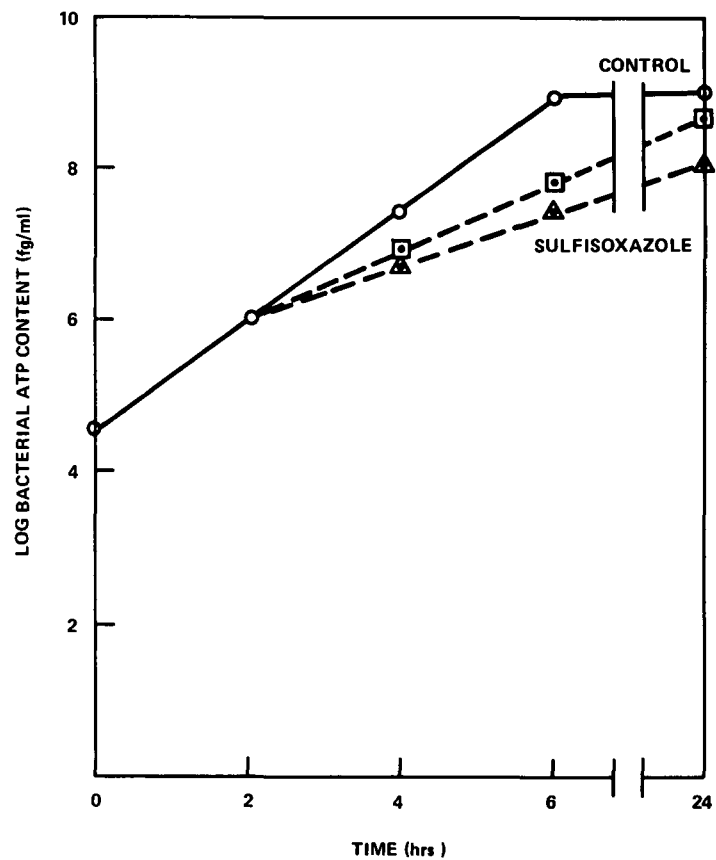
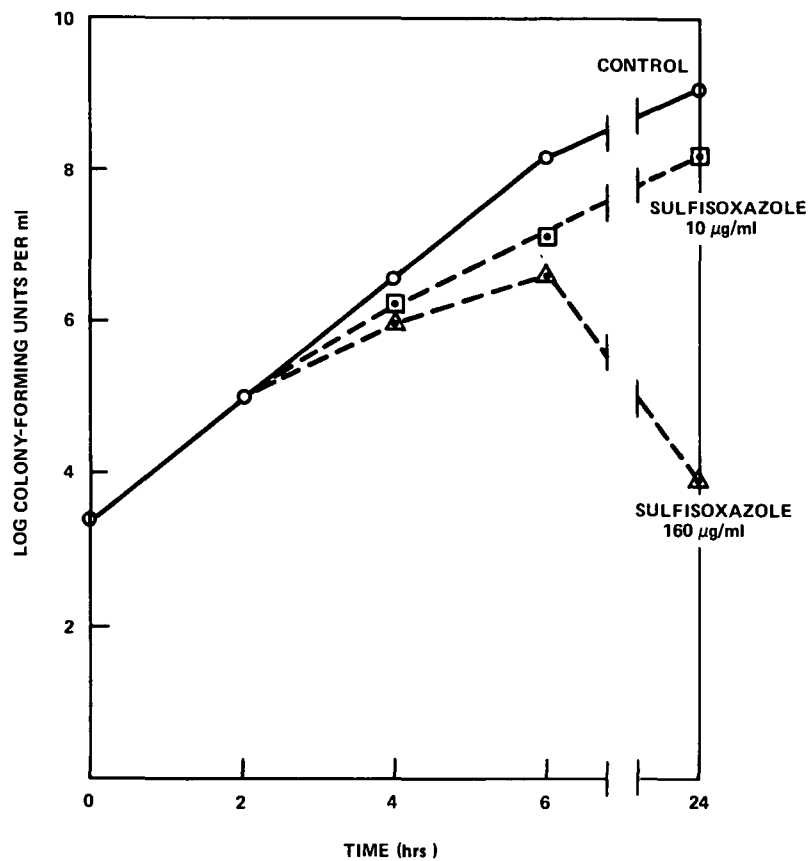


Figure 4-3. Growth inhibition of *E. coli* ATCC 25922 by sulfisoxazole at 10 µg/ml (□—□) and 160 µg/ml (Δ—Δ); Comparison of viable counts and bacterial ATP content.

Nalidixic Acid

In our preliminary work, the ATP index consistently gave false resistant results (figure 4-4). This is not surprising because analysis of the mode of action of this antimicrobial agent has shown that it selectively inhibits bacterial DNA synthesis without significantly altering RNA, protein, or lipid synthesis (Goss et al., 1964). Nalidixic acid also has little or no effect on respiration with glucose as a substance. Thus, turbidometric and radiometric methods are also likely to show false resistance to this agent.

HETEROGENEITY OF THE BACTERIAL INOCULUM

There may be a substantial portion of a bacterial inoculum, on the order of 10^6 colony-forming units/ml, that are immediately susceptible to the effects of the antibiotic, while the residual population is still able to recover to indicate resistance by the usual methods requiring overnight incubation.

This phenomenon is also illustrated in uncommon instances where, despite an apparent zone of inhibition in the susceptible range by agar diffusion, several colonies are seen within the zone. Provided purity of the strain can be confirmed, this must be interpreted as resistance (Bauer et al., 1966). Rapid susceptibility methods are likely to report them as falsely sensitive, as in the case of *Enterobacter* versus colistin.

A variant of this effect is illustrated by *Enterococcus* versus clindamycin, where as little as 0.063 of the MIC markedly prolongs the lag phase resulting in false sensitive interpretation (figure 4-5).

SUMMARY

We have attempted to give adequate explanations for the discrepancies that we have found between the ATP index and agar disc-diffusion techniques. Most, if not all of these, are shared by other techniques for rapid determination of microbial susceptibility.

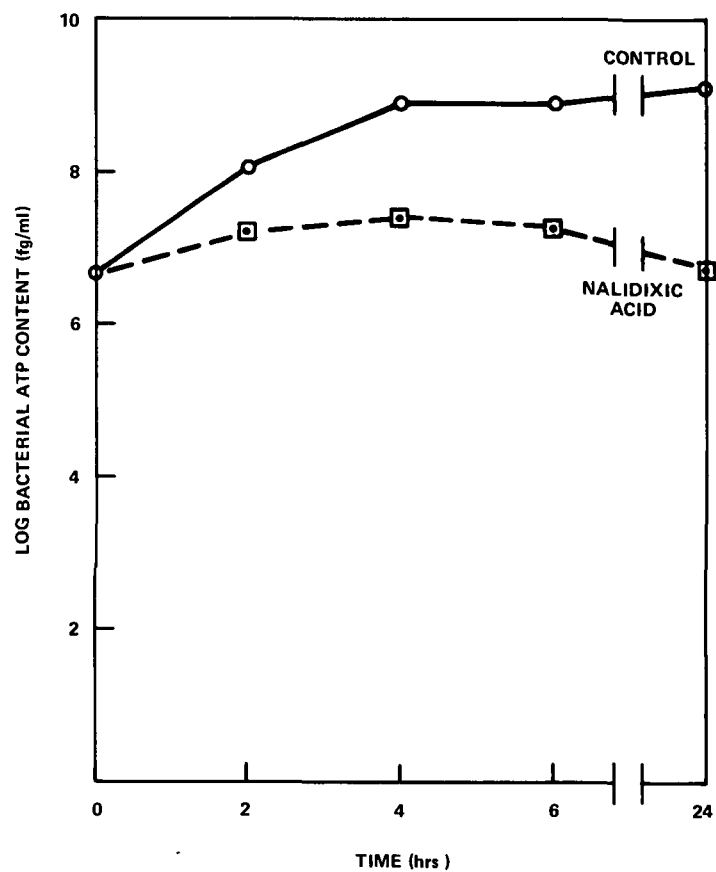
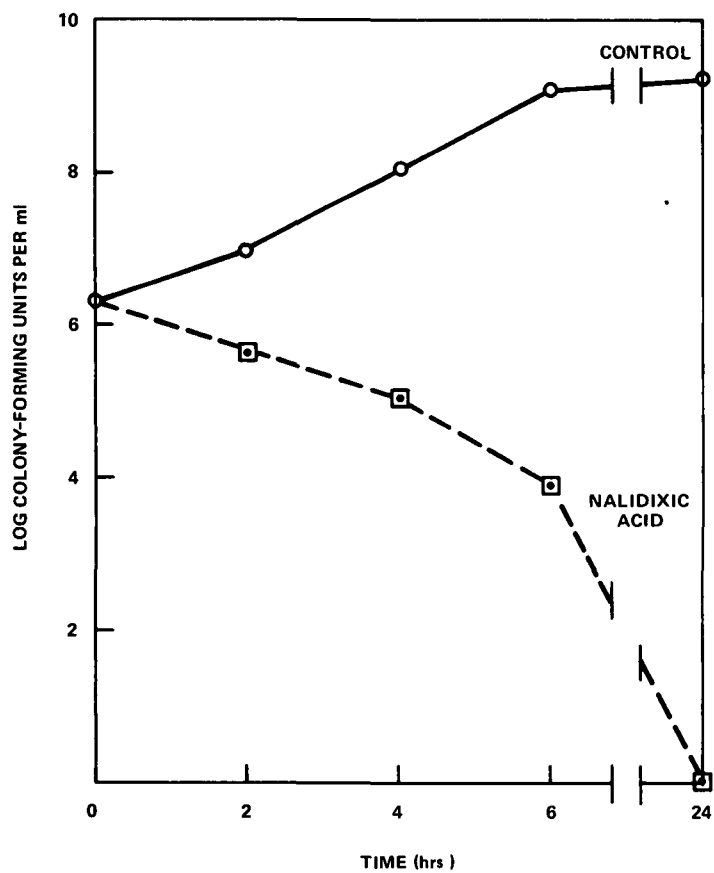


Figure 4-4. Growth inhibition of *E. coli* ATCC 25922 by nalidixic acid at 12.5 $\mu\text{g/ml}$ ($\square$ — $\square$); Comparison of viable counts and bacterial ATP content.

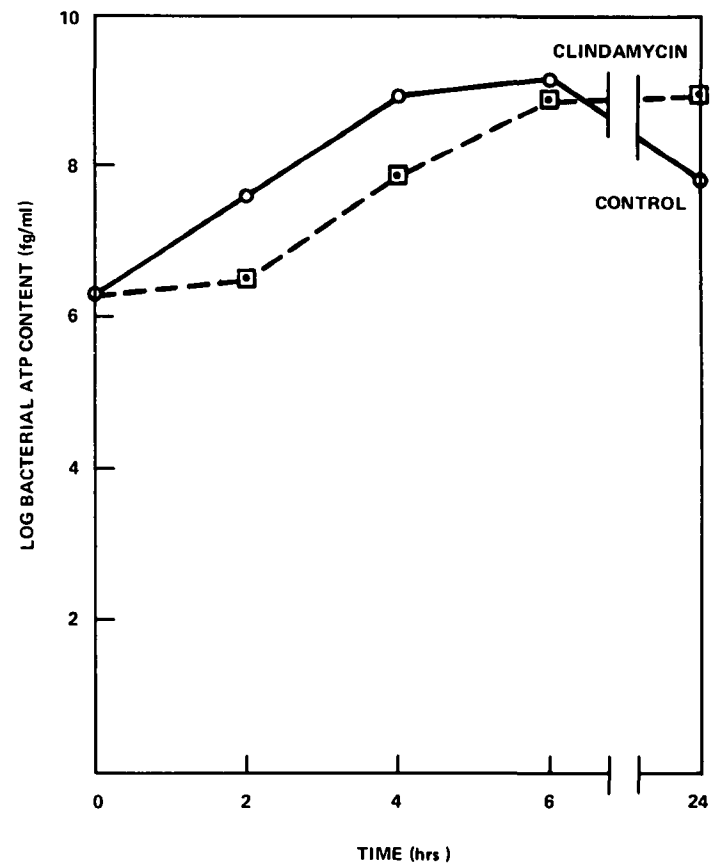
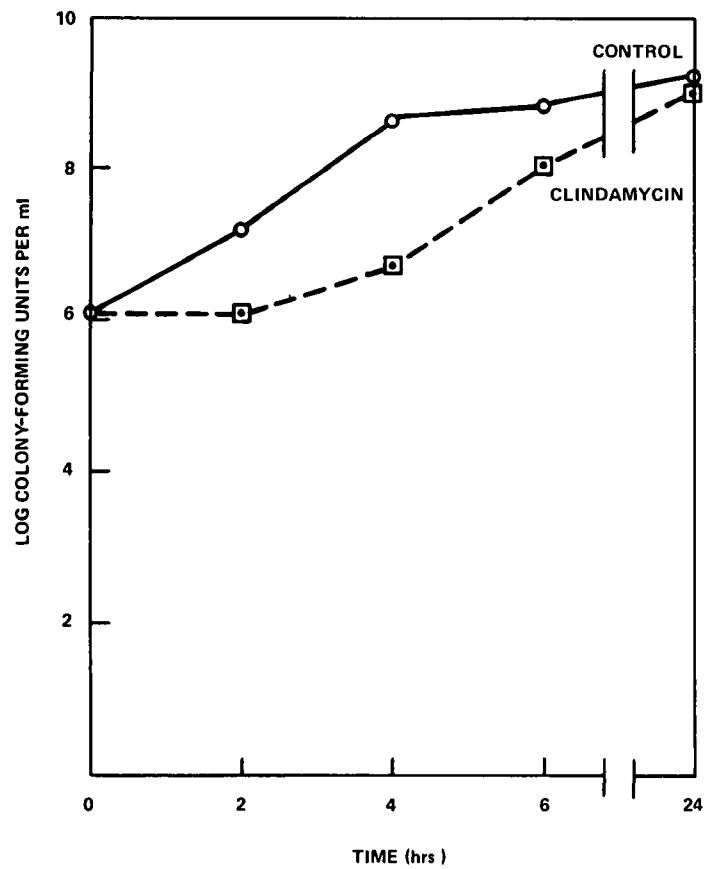


Figure 4-5. Growth inhibition of *Streptococcus faecalis* (*Enterococcus*) 04390 by clindamycin at 8 μ g/ml ($\square$ — $\square$); Comparison of viable counts and bacterial ATP content.

Table 4-1
Comparison of Results Obtained by ATP Index, Agar Diffusion
(Kirby-Bauer), and Tube Dilution MIC

Antibiotic ($\mu\text{g/ml}$)	<i>S. aureus</i> ATCC 25923				<i>S. aureus</i> S-187				<i>S. epidermidis</i> 05995			
	K-B (mm)	MIC ($\mu\text{g/mg}$)	ATP Index	Interpretation *	K-B (mm)	MIC ($\mu\text{g/ml}$)	ATP Index	Interpretation*	K-B (mm)	MIC ($\mu\text{g/ml}$)	ATP Index	Interpretation *
Penicillin G (8) (0.15)	32.5	<0.125	-0.86 -0.70	S S	ND 14.5	ND ND	ND +0.53	ND R	21	1.0	-0.46	S
Ampicillin (3)	31	<0.125	-0.17	S	ND	ND	ND	ND	25	0.5	-0.02	S
Nafcillin (0.6)	19.5	ND	-0.79	S	19.5	ND	-0.39	S	ND	ND	ND	ND
Carbenicillin (128)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Cephalothin (16)	32.5	<0.125	-0.38	S	ND	ND	ND	ND	35	0.25	-0.24	S
Tetracycline (6)	25.5	0.5	-0.13	S	ND	ND	ND	ND	0	>128	+1.01	R
Erythromycin (4)	20	0.25	0.00	S	ND	ND	ND	ND	29.5	0.25	+0.08	S
Clindamycin (2)	26	<0.125	-0.26	S	ND	ND	ND	ND	29	<0.125	-0.01	S
Centamlein (6)	24	0.25	-0.07	S	ND	ND	ND	ND	30	0.125	+0.04	S
Nitrofurantoin (50)	18.5	16	-0.05	S	ND	ND	ND	ND	25	4	-0.12	S
Colistin (8)	0	>128	+0.88	R	ND	ND	ND	ND	0	>128	+0.91	R
Chloramphenicol (12.5)	24	4	-0.28	S	ND	ND	ND	ND	27.5	4	-0.24	S

*Where R = resistant, S = sensitive, ND = not done

Table 4-1 (Continued)

Antibiotic ($\mu\text{g/ml}$)	<i>Proteus mirabilis</i> 04583				<i>Pseudomonas aeruginosa</i> 04275				<i>Enterococcus</i> 04390			
	K-B (mm)	MIC ($\mu\text{g/ml}$)	ATP Index	Interpretation	K-B (mm)	MIC ($\mu\text{g/ml}$)	ATP Index	Interpretation	K-B (mm)	MIC ($\mu\text{g/ml}$)	ATP Index	Interpretation
Penicillin G (8)	20	2.5	-0.10 +0.82	S/S-R	0	>80	+0.84	R	18	2.5	-0.35	S
Ampicillin (8)	26.5	1.0	+0.53	S/R	0	>128	+0.82	R	22	1.0	-0.54	S
Nafcillin (0.6)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Carbenicillin (128)	30	2	+0.10	S	19	128	+0.68	S/R	ND	ND	ND	ND
Cephalothin (16)	20	4	-0.03	S	0	>128	+0.93	R	14	32	+0.78	R
Tetracycline (6)	0	128	+0.73	R	0	64	+0.47	R	20	1.0	-0.04	S
Erythromycin (4)	0	>128	+0.95	R	0	>128	+0.95	R	26	<0.125	+0.18	S
Clindamycin (2)	0	>128	+0.99	R	0	>128	+0.90	R	0	8	+0.01	R/S
Gentamicin (6)	22	8	-0.20	S	20.5	2	-0.38	S	13	64	+0.88	R
Nitrofurantoin (50)	11	128	+0.45	R	0	>128	+0.98	R	23	8	+0.25	S
Colistin (8)	0	>128	+0.89	R	16	8	-0.58	S	0	>128	+0.69	R
Chloramphenicol (12.5)	28.0	4	-0.15	S	14	64	+0.46	R	22.0	8	-0.05	S

Table 4-1 (Continued)

Antibiotic ($\mu\text{g/ml}$)	<i>Escherichia coli</i> ATCC 25922				<i>Klebsiella</i> 07220				<i>Enterobacter</i> 05248			
	K-B (mm)	MIC ($\mu\text{g/ml}$)	ATP Index	Interpretation	K-B (mm)	MIC ($\mu\text{g/ml}$)	ATP Index	Interpretation	K-B (mm)	MIC ($\mu\text{g/ml}$)	ATP Index	Interpretation
Penicillin G (8)	0	80	+0.99	R	0	>80	+0.99	R	0	>80	+1.00	R
Ampicillin (8)	19	8	-0.33	S	0	>128	+0.93	R	0	>128	+0.93	R
Nafcillin (0.6)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Carbenicillin (128)	28	32	-0.27	S	ND	ND	ND	ND	ND	ND	ND	ND
Cephalothin (16)	19	32	-0.30	S	21	16	-0.46	S	0	>128	+0.97	R
Tetracycline (8)	20	4	+0.13	S	19	8	+0.07	S	19	8	+0.04	S
Erythromycin (4)	10.5	64	+0.92	R	11.0	128	+0.98	R	0	>128	+0.98	R
Clindamycin (2)	0	128	+0.96	R	0	>128	+0.94	R	0	>128	+0.94	R
Gentamicin (6)	22	4	-0.43	S	22	2	-0.39	S	24	2	-0.40	S
Nitrofurantoin (50)	23.5	16	-0.12	S	13	128	+0.49	R	22	32	-0.16	S
Colistin (8)	15.5	8	-0.76	S	16	16	-0.73	S	13	>128	-0.72	R/S
Chloramphenicol (12.5)	25.5	8	+0.01	S	29.5	16	-0.01	S	26.0	16	-0.12	S

SECTION 5

INTERIM PROGRESS REPORT, JANUARY 1975: DIRECT APPLICATION OF ATP ANTIMICROBIAL SUSCEPTIBILITY TESTING TO URINE SPECIMENS

INTRODUCTION

In July 1974, attention turned to the major objective of applying the luciferase antimicrobial susceptibility methods directly to urine specimens. The effort included:

- Evaluation of centrifugation to isolate organisms from urine specimens for subsequent susceptibility tests
- Evaluation of the effect of urine (material accompanying the bacteria in the sediment) on the luciferase antimicrobial susceptibility methods
- Preliminary evaluation of the method with clinical urine samples
- Use of an ATP assay (the long centrifugation method) suited for detection of bacteriurias to establish appropriate inoculum size for susceptibility testing
- Addition of kanamycin and streptomycin to antibiotics being evaluated
- Modification of certain technical aspects of methods to increase efficiency and accuracy
- Evaluation of the modified method, incorporating the last three efforts listed above, with clinical urine samples, including a detailed analysis of errors.

EVALUATION OF CENTRIFUGATION TO ISOLATE ORGANISMS FROM URINE SPECIMENS FOR SUBSEQUENT SUSCEPTIBILITY TESTING

The susceptibility testing method employing the firefly luciferase ATP assay requires a certain inoculum size and the presence of organisms in growth media for consistent growth patterns from sample to sample. These requirements were the initial considerations in applying the method directly to urine samples. The concept of using the urine itself as growth media was first considered. Unfortunately, at least two physiochemical parameters of urine greatly alter growth of pathogens in urines. Others have disclosed the effects of pH and osmolality on mean generation time of *E. coli* as shown in figures 5-1 and 5-2.

Perhaps pH could be adjusted, but osmolality would be difficult to adjust for each sample. Clearly the best approach would be to separate the organisms from the urine. The effects of pH and osmolality could be eliminated, and the inoculum size could be controlled.

Centrifugation and filtration were considered. Filtration of the volume necessary to achieve an appropriate inoculum size is difficult. Centrifugation of urine has been shown previously to give workable recovery of organisms (unpublished work performed at Goddard Space Flight Center).

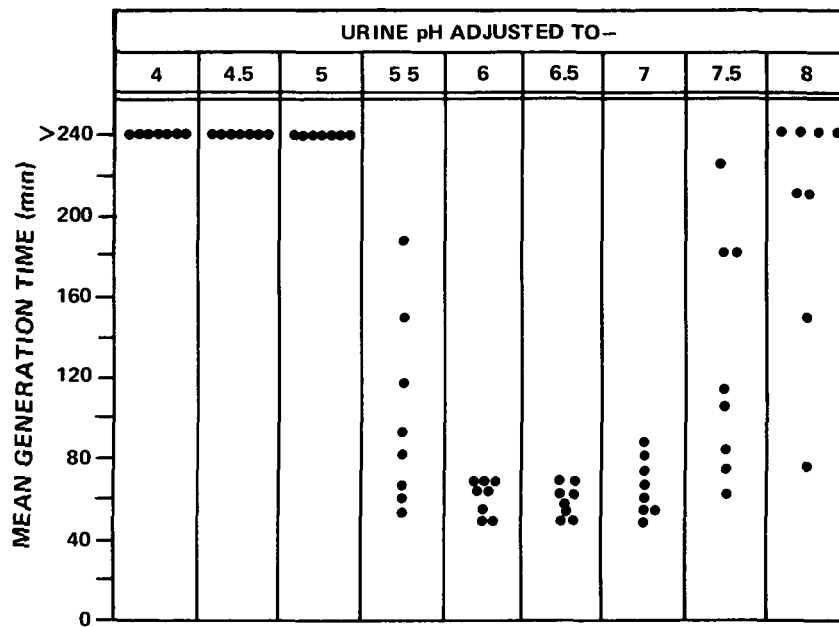


Figure 5-1. Effect of urinary pH on the growth rate of *E. coli*.

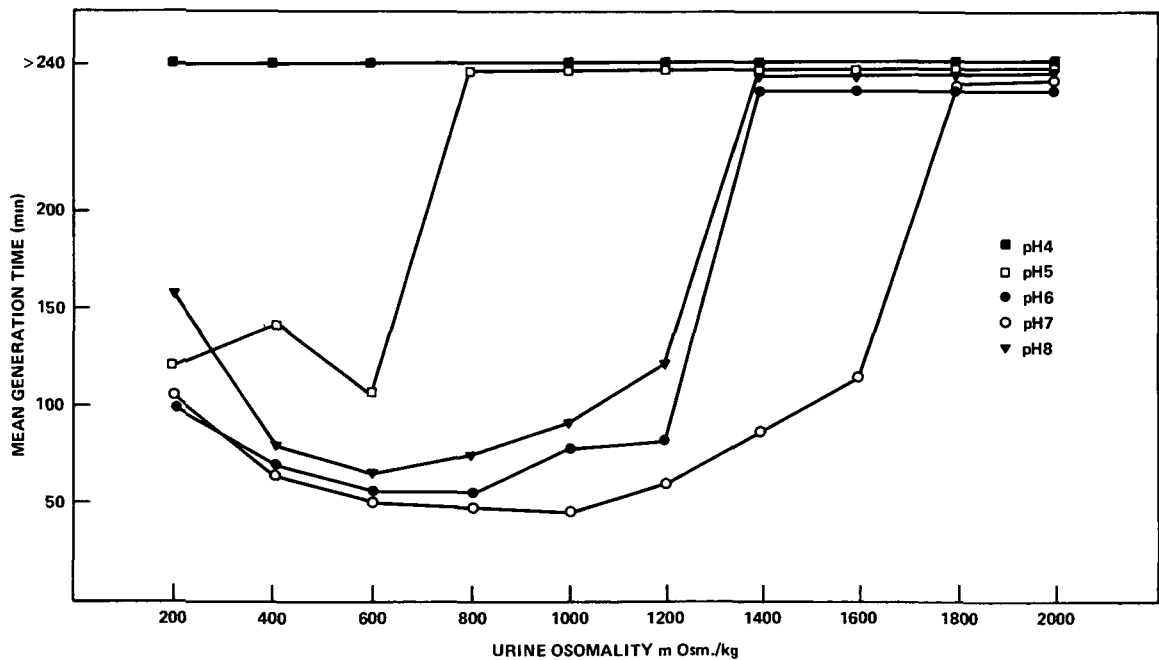


Figure 5-2. Effect of variation of urinary pH and osmolality on the growth rate of *E. coli*.

Centrifugation was evaluated as a means to isolate organisms from the urine. Experiments were designed to allow the additional evaluation of the ability to achieve and use an inoculum size of approximately 1×10^5 organisms/ml which can be measured by the nonconcentrated procedure without malic acid for luciferase assay of bacterial ATP (method employed by Vellend in pure species work and described on page 8). With a three-fold concentration by centrifugation, this inoculum size could be achieved from urines with thirty thousand organisms per ml, the lowest bacterial count considered to represent a significant bacteriuria at the Microbiology Laboratory at NEMCH.

Experimental Plan

Sterile urine was infected with various reference organisms to give 30,000 organisms/ml. Forty-five ml of infected urine were centrifuged at 8000 RCF $\times$ G for 15 minutes at 4°C. The supernate was decanted, and the tube allowed to drain in the upright position approximately 30 seconds. The bacterial pellet was reconstituted in 15 ml of TSB to give a final concentration of 90,000 bacteria/ml. This specimen was then handled exactly as Dr. Vellend has described for his pure culture work using the nonconcentrated procedure without malic acid for luciferase assay. After preincubation of the sample at 37°C for 30 minutes, aliquots of 0.9 ml were placed into each of 14 tubes. Tube A contained 0.1 ml of sterile water and served as A_0 and growth control for all antibiotics except nitrofurantoin. Tube A_{Nitro} contained 0.2 ml of TSB and served as A_0 and growth control for nitrofurantoin. Tube B_{1-12} contained 0.1 ml of an antibiotic solution in sterile water to yield a final concentration shown in the following list. B_{Nitro} contained 0.2 ml of an antibiotic solution in TSB to yield a final concentration of 50 $\mu\text{g/ml}$ of nitrofurantoin.

Antimicrobial Agents Used in Preliminary Studies

<u>Drugs</u>	<u>Concentration ($\mu\text{g/ml}$)</u>
Penicillin	8
Ampicillin	8
Nafcillin	0.6
Carbenicillin	128
Cephalothin	16
Tetracycline	6
Chloramphenicol	12.5
Clindamycin	2
Erythromycin	4
Gentamicin	6

<u>Drugs</u>	<u>Concentration ($\mu\text{g/ml}$)</u>
Colistin	8
Kanamycin	16
Streptomycin	10
Nitrofurantoin	50

From the control tubes A and A_{Nitro} , 0.5 ml of the cultures were assayed immediately for ATP (A_0). All tubes were then incubated at 37°C for 2.5 hours. After incubation 0.5-ml samples were assayed for ATP.

All ATP assays were performed using the following method:

1. Pipette 0.5 ml sample of broth culture into a sterile polypropylene tube (17 × 100 mm).
2. Add 0.1 ml Apy-Ca-TX, vortex well, and wait 5 minutes.
3. Add 0.1 ml of 1.5 N nitric acid to extract bacterial ATP, vortex well, and wait 5 minutes.
4. Add 4.3 ml of sterile deionized water and mix well.
5. Using a disposable tuberculin syringe, inject 0.1 ml of the mixture into the luciferase reagent (rehydrated in 0.25 M TRIS, 0.01 M MgSO_4 , at pH 8.0).
6. Measure light response in photometer.

Samples

The urine samples were first-voided specimens from a healthy volunteer. Sterility was established by usual culture techniques. Urinalysis revealed specific gravities that ranged from 1.016 to 1.021. A range of pH from 5.0 to 6.0 was determined by Bili-labstix® (Ames). Chemistries by Bili-labstix® were negative for blood, bilirubin, ketones, glucose, and protein. Microscopic examination of sediment revealed only an occasional white blood cell (wbc), red blood cell (rbc), or crystal.

The reference strains were those previously used to validate the ATP technique. Two reference strains were provided by the Food and Drug Administration:

(1) *Staphylococcus aureus* ATCC 25923 and (2) *Escherichia coli* ATCC 25922. Other species used were strains isolated from urine specimens submitted to the diagnostic Microbiology Laboratory at the NEMCH: *Klebsiella aerogenes* 07220, *Enterobacter cloacae* 05248, *Proteus mirabilis* 04583, *Pseudomonas aeruginosa* 04275, *Enterococcus* (*Streptococcus fecalis*) 04390, and *Staphylococcus epidermidis* 05995.

Table 5-1

**Comparison of Antibiotic Susceptibility Results by Standard Kirby-Bauer Test
with Results Using ATP Method and Proposed Centrifugation
Procedure to Isolate Bacteria from Urine**

Antibiotic	Index	ATP Interpretation	KB	KB Interpretation	Usable	Disagreement with KB
30,000 <i>S aureus</i>						
$A_0 = 2.86 \times 10^5, A_{2.5} = 3.05 \times 10^6$						
Penicillin	-0.03	S	32.5	S	-	(false R)
Ampicillin	-0.02	S	31	S		
Nafcillin	-0.04	S	19.5	S		
Carbenicillin	-0.03	S	-	-		
Cephalothin	+0.08	R	32.5	S		
Tetracycline	-0.01	S	25.5	S		
Chloramphenicol	-0.01	S	24	S		
Clindamycin	-0.00	S	26	S		
Erythromycin	+0.01	S	28	S		
Gentamicin	-0.01	S	24	S		
Colistin	+0.44	R	0	R		
Nitrofurantoin	-0.01	S	18.5	S		
30,000 <i>Enterococcus</i>						
$A_0 = 1.30 \times 10^6, A_{2.5} = 8.07 \times 10^7$						
Penicillin	-0.01	S	18	I	-	(false S)
Ampicillin	-0.01	S	22	S		
Nafcillin	+0.87	R	-	-		
Carbenicillin	-0.01	S	-	-		
Cephalothin	+0.19	R	14	R		
Tetracycline	+0.01	S	20	S		
Chloramphenicol	+0.00	S	22	S		
Clindamycin	+0.01	S	0	R		
Erythromycin	+0.01	S	26	S		
Gentamicin	+0.25	R	13	S		
Colistin	+1.07	R	0	R		
Nitrofurantoin	+0.04	S	23	S		
30,000 <i>P mirabilis</i>						
$A_0 = 6.86 \times 10^5, A_{2.5} = 4.19 \times 10^7$						
Penicillin	-0.01	S	20	I	-	
Ampicillin	-0.01	S	26.5	S		
Nafcillin	+0.87	R	-	-		
Carbenicillin	-0.01	S	30	S		
Cephalothin	+0.00	S	20	S		
Tetracycline	+0.46	R	0	R		
Chloramphenicol	-0.00	S	28	S		
Clindamycin	+0.89	R	0	R		
Erythromycin	+0.80	R	0	R		
Gentamicin	-0.01	S	22	S		
Colistin	+0.56	R	0	R		
Nitrofurantoin	+0.39	R	11	R		
30,000 <i>E coli</i>						
$A_0 = 0.33 \times 10^5, A_{2.5} = 1.13 \times 10^7$						
Penicillin	+0.80	R	0	R		
Ampicillin	-0.00	S	19	S		
Nafcillin	+0.78	R	-	-		
Carbenicillin	-0.00	S	28	S		
Cephalothin	-0.00	S	19	S		
Tetracycline	+0.01	S	20	S		
Chloramphenicol	+0.01	S	25.5	S		
Clindamycin	+0.75	R	0	R		
Erythromycin	+0.75	S	10.5	R		
Gentamicin	-0.00	S	22	S		
Colistin	-0.00	S	15.5	S		
Nitrofurantoin	-0.01	S	23.5	S		

Table 5-1 (Continued)

Antibiotic	Index	ATP Interpretation	KB	KB Interpretation	Usable	Disagreement with KB
30,000 <i>Klebsiella pneumoniae</i>						
$A_0 = 4.64 \times 10^5$, $A_{2.5} = 4.72 \times 10^7$						
Penicillin	+1 33	R	0	R		
Ampicillin	+1.04	R	0	R		
Nafcillin	+1.04	R	-	-	-	
Carbenicillin	+1.10	R	-	-	-	
Cephalothin	-0.01	S	21	S		
Tetracycline	+0 01	S	19	S		
Chloramphenicol	+0.01	S	29.5	S		
Clindamycin	+1.13	R	0	R		
Erythromycin	+1 23	R	11	R		
Gentamicin	-0 01	S	22	S		
Collistin	-0 00	S	16	S		
Nitrofurantoin	+0 14	R	13	R		
30,000 <i>Enterobacter</i>						
$A_0 = 4.1 \times 10^5$, $A_{2.5} = 5.30 \times 10^6$						
Penicillin	+1 11	R	0	R		
Ampicillin	+1 21	R	0	R		
Nafcillin	+1 22	R	-	-	-	
Carbenicillin	-0 03	S	-	-	-	
Cephalothin	+1.18	R	0	R		
Tetracycline	+0 01	S	19	S		
Chloramphenicol	-0.01	S	26	S		
Clindamycin	+1.01	R	0	R		
Erythromycin	+0.83	R	0	R		
Gentamicin	-0 05	S	24	S		
Collistin	+0 88	R	13	S		(false R)
Nitrofurantoin	+0 03	S	22	S		

Results

Table 5-1 reports the results for six reference strains. The reference organism and light units obtained for growth controls A_0 and $A_{2.5}$ are given initially in each case. The column marked Index lists the ATP index of inhibition for each antibiotic calculated as follows:

$$\text{ATP Index} = \frac{B - A_0}{A_{2.5} - A_0}$$

where A_0 = light units for growth control after 0.5 hour preincubation at 37°C; $A_{2.5}$ = light units for growth control after 2.5 hours of incubation at 37°C; and B = light units for sample containing appropriate antibiotic concentration after 2.5 hours of incubation at 37°C.

The column marked ATP Interpretation gives the interpretation of an index according to the criteria:

Resistant (R) > 0.05

Sensitive (S) ≤ 0.05

The column marked KB gives zone diameters in millimeters of the Kirby-Bauer agar diffusion technique for the organism being tested. Technique and interpretation of resulting values were carried out according to the standards recommended by the National Committee for Clinical Laboratory Standards in August 1972.

The far right column notes when ATP results disagreed with Kirby-Bauer results. If the Kirby-Bauer interpretation was sensitive and the ATP interpretation resistant, the ATP result was labeled as false-resistant. If the Kirby-Bauer interpretation was resistant and the ATP sensitive, the ATP result was considered false-sensitive.

Discussion

Good correlation between the Kirby-Bauer and ATP techniques was achieved under these conditions. Of 63 determinations, only 4 disagreements were found, giving a 93.6-percent correlation.

Two major points were established. First, centrifugation could be used to isolate organisms from urine, and the material accompanying the bacteria in the sediment (in this case amorphous material, occasional crystals, white blood cells, and red blood cells) did not interfere with subsequent antibiotic sensitivity testing. Second, an inoculum of approximately 1×10^5 could be used successfully with the nonconcentrated procedure without malic acid for luciferase assay. Success at an inoculum of 1×10^6 had been established in earlier studies. Furthermore, this inoculum size could be achieved from urines with 30,000 organisms/ml by a 3-fold concentration. This indicated that even a minimally significant bacteriuria of 30,000 organisms/ml could be evaluated successfully.

EVALUATION OF THE EFFECT OF URINE (MATERIAL ACCOMPANYING THE BACTERIA IN THE SEDIMENT) ON THE LUCIFERASE ANTIMICROBIAL SUSCEPTIBILITY METHODS

The concern prompting this study was that the urinary sediment could contain material that would interfere with the growth of bacteria, with antibiotic activity, or with the ATP assay itself. Any one of these influences could hinder the sensitivity testing and reduce correlation with the Kirby-Bauer technique.

Experimental Plan

To evaluate this problem, sterile urines (no growth on overnight plates) were obtained from patients with various clinical conditions or metabolic disorders, who were taking a variety of medications, including antibiotics. The urines were artificially infected with 30,000 organisms/ml of reference strain *E. coli*. The procedure was carried out exactly as described above using the nonconcentrated procedure without malic acid.

Patients had a variety of diagnoses which included pneumonia, serum hepatitis, rectal abscess, hypothyroidism, inflammatory bowel disease, carcinoma of bladder, lymphoma, endometrial carcinoma, floppy mitral valve, and abdominal aneurysm. Metabolic disorders included diabetes mellitus in one case and hypercholesterolemia in another.

Medications included isoniazid, folic acid, pyridoxine, codeine phosphate, prochlorperazine, acetaminophen, disizepam, procabazine, diphenhydramine hydrochloride, flurazepam, hydrochlorothiazide, spironoactone, clofibrate, prednisone, methyldopa, indomethacin, and dioctyl sodium sulfosuccinate.

Six patients were on antibiotics, which included two on cefazolin sodium, two on cephalothin, one on penicillin, and one on both oxacillin and ampicillin. Urinalysis revealed that specific gravities ranged from 1.005 to 1.035, and pH ranged from 5.0 to 7.0. Chemistries by Bili-labstix® revealed that two patients had 1+ protein. Sediment exam revealed amorphous debris in many cases, rare white blood counts in five cases, rare epithelial cells in three cases, one case with 5.7×10^3 renal tubular cells, and one case with 2.0×10^4 white blood cells/ml.

Results

Table 5-2 is a tabulation of the results obtained during this evaluation. The table is similar in format to table 5-1 and the previous explanation applies.

There was good correlation between the Kirby-Bauer and the ATP method with urines from patients who were not on antibiotics. Sample numbers 2, 3, 4, 8, 9, 11, 12, 13, and 15 were from patients who were not taking antibiotics, and there was only one point of disagreement (erythromycin, false S, sample 9) in 54 determinations representing a 98-percent correlation.

There was poor correlation found with three samples. Samples numbers 7 and 10 had a 50-percent correlation, and sample number 1 agreed at only one point. In all these cases, the

Table 5-2

Results of the Evaluation of the Effect of Urine on the
- Luciferase Antimicrobial Susceptibility Methods with
30,000 *Escherichia coli* per ml

Antibiotic	Index	ATP Interpretation	Kirby-Bauer	Kirby-Bauer Interpretation	Disagreement with Kirby-Bauer
Sample No. 1 $A_0 = 0.14 \times 10^5$, $A_2 = 0.36 \times 10^5$					
Ampicillin	+0.18	R	19	S	false R
Cephalothin	+1.00	R	19	S	false R
Tetracycline	+3.59	R	20	S	false R
Chloramphenicol	+7.23	R	25.5	S	false R
Enythromycin	+1.32	R	10.5	R	
Gentamicin	+6.63	R	22	S	false R
Sample No. 2 $A_0 = 2.13 \times 10^5$, $A_2 = 2.39 \times 10^7$					
Ampicillin	-0.01	S	19	S	
Cephalothin	-0.01	S	19	S	
Tetracycline	+0.01	S	20	S	
Chloramphenicol	+0.00	S	25.5	S	
Enythromycin	+0.95	R	10.5	R	
Gentamicin	-0.00	S	22	S	
Sample No. 3 $A_0 = 1.91 \times 10^5$, $A_2 = 8.83 \times 10^6$					
Ampicillin	+0.00	S	19	S	
Cephalothin	+0.01	S	19	S	
Tetracycline	+0.04	S	20	S	
Chloramphenicol	+0.03	S	25.5	S	
Enythromycin	+1.07	R	10.5	R	
Gentamicin	+0.02	S	22	S	
Sample No. 4 $A_0 = 0.55 \times 10^5$, $A_2 = 5.09 \times 10^6$					
Ampicillin	-0.00	S	19	S	
Cephalothin	-0.00	S	19	S	
Tetracycline	+0.03	S	20	S	
Chloramphenicol	+0.03	S	25.5	S	
Enythromycin	+0.86	R	10.5	R	
Gentamicin	-0.00	S	22	S	
Sample No. 5 $A_0 = 0.20 \times 10^5$, $A_2 = 2.17 \times 10^7$					
Ampicillin	+0.00	S	18	S	
Cephalothin	+0.01	S	19	S	
Tetracycline	+0.01	S	20	S	
Chloramphenicol	+0.01	S	25.5	S	
Enythromycin	+0.80	R	10.5	R	
Gentamicin	+0.00	S	22	S	
Sample No. 6 $A_0 = 3.65 \times 10^5$, $A_2 = 5.94 \times 10^7$					
Ampicillin	-0.00	S	19	S	
Cephalothin	-0.00	S	19	S	
Tetracycline	+0.01	S	20	S	
Chloramphenicol	+0.00	S	25.5	S	
Enythromycin	+0.93	R	10.5	R	
Gentamicin	-0.00	S	22	S	
Sample No. 7 $A_0 = 3.06 \times 10^5$, $A_2 = 1.49 \times 10^5$					
Ampicillin	-0.88	S	19	S	
Cephalothin	-0.85	S	19	S	
Tetracycline	+0.31	R	20	S	false R
Chloramphenicol	+0.06	R	25.5	S	false R
Enythromycin	+0.17	S	10.5	R	false R
Gentamicin	-0.79	S	22	S	

Table 5-2 (Continued)

Sample No. 8 $A_0 = 3.75 \times 10^6$, $A_{2.5} = 5.88 \times 10^7$					
Ampicillin	+0.01	S	19	S	
Cephalothin	-0.05	S	19	S	
Tetracycline	+0.02	S	20	S	
Chloramphenicol	-0.00	S	25.5	S	
Enythromycin	-0.52	R	10.5	R	
Gentamicin	-0.06	S	22	S	
Sample No. 9 $A_0 = 1.16 \times 10^6$, $A_{2.5} = 7.92 \times 10^7$					
Ampicillin	-0.01	S	19	S	
Cephalothin	-0.01	S	19	S	
Tetracycline	+0.01	S	20	S	
Chloramphenicol	-0.00	S	25.5	S	
Enythromycin	+0.03	R	10.5	R	false S
Gentamicin	-0.01	S	22	S	
Sample No. 10 $A_0 = 3.69 \times 10^5$, $A_{2.5} = 1.45 \times 10^6$					
Ampicillin	+0.11	R	19	S	false R
Cephalothin	+0.08	R	19	S	false R
Tetracycline	+0.05	S	20	S	
Chloramphenicol	+0.10	R	25.5	S	false R
Enythromycin	+0.33	R	10.5	R	
Gentamicin	-0.04	S	22	S	
Sample No. 11 $A_0 = 1.24 \times 10^6$, $A_{2.5} = 3.64 \times 10^7$					
Ampicillin	-0.02	S	19	S	
Cephalothin	-0.02	S	19	S	
Tetracycline	-0.00	S	20	S	
Chloramphenicol	-0.00	S	25.5	S	
Enythromycin	+1.02	R	10.5	R	
Gentamicin	-0.03	S	22	S	
Sample No. 12 $A_0 = 6.05 \times 10^5$, 1.23×10^8 \					
Ampicillin	-0.00	S	19	S	
Cephalothin	-0.00	S	19	S	
Tetracycline	+0.00	S	20	S	
Chloramphenicol	+0.00	S	25.5	S	
Enythromycin	+0.09	R	10.5	R	
Gentamicin	-0.00	S	22	S	
Sample No. 13 $A_0 = 5.40 \times 10^5$, $A_{2.5} = 9.80 \times 10^7$					
Ampicillin	-0.01	S	19	S	
Cephalothin	-0.01	S	19	S	
Tetracycline	+0.01	S	20	S	
Chloramphenicol	+0.00	S	25.5	S	
Enythromycin	+0.77	R	10.5	R	
Gentamicin	-0.01	S	22	S	
Sample No. 14 $A_0 = 7.37 \times 10^5$, $A_{2.5} = 1.33 \times 10^8$					
Ampicillin	-0.03	S	19	S	
Cephalothin	-0.03	S	19	S	
Tetracycline	+0.00	S	20	S	
Chloramphenicol	+0.00	S	25.5	S	
Enythromycin	+0.86	R	10.5	R	
Gentamicin	-0.03	S	22	S	
Sample No. 15 $A_0 = 4.57 \times 10^5$, $A_{2.5} = 1.24 \times 10^8$					
Ampicillin	-0.01	S	19	S	
Cephalothin	-0.01	S	19	S	
Tetracycline	-0.00	S	20	S	
Chloramphenicol	-0.00	S	25.5	S	
Enythromycin	+0.95	R	10.5	R	
Gentamicin	-0.01	S	22	S	

patient was taking an antibiotic. In sample 1, the patient was on both ampicillin and oxacillin, in sample 7, the patient was on cephalothin, and in sample 10, the patient was on penicillin. In each case there was little or no growth in the growth control as seen by comparing A_0 and $A_{2.5}$ values. Three samples from patients on antibiotics gave 100-percent correlation: sample 5 was from a patient on cephalothin and samples 6 and 14 were from patients on cefazolin.

Discussion

The excellent correlation obtained when patients were not on antibiotics indicates that material accompanying the bacteria in the sediment does not interfere with sensitivity tests. A variety of medications were taken by patients whose urine samples gave 100-percent correlation; these urine samples also exhibited a large range of osmolalities and pH.

In the case of the samples which disagreed, no other factor except the antibiotic usage by the patient was identified. Antibiotic sensitivity tests by the ATP method were unsuccessful because little or no growth of the organisms in the growth control occurred. Apparently, even short exposure of the *E. coli* to the antibiotic in the urine before centrifugation interfered with the subsequent growth after transfer to the broth.

Clearly, this is an artificial system. In the clinical situation, a patient would not develop a urinary tract infection with an organism sensitive to an antibiotic being administered. If a urinary tract infection did arise in this situation, the organisms would be resistant, growth would occur in the growth control, and ATP sensitivity testing could probably be carried out.

PRELIMINARY EVALUATION OF METHOD ON CLINICAL URINE SAMPLES

The foregoing work had established that: (1) centrifugation could be used to isolate bacteria from the urine; (2) an inoculum of 1×10^5 organisms/ml gave satisfactory results and could be achieved from urines with 30,000 organisms/ml; and (3) urine and material contained in the urine did not interfere with susceptibility tests (the only exception being when the urine contained an antibiotic to which the organism was sensitive, a situation which should not be encountered clinically).

At this point, it was feasible to begin a preliminary clinical trial. It would be desirable to obtain urines known to be infected with a single organism at a level of 30,000 organisms/ml or greater. A method to obtain such samples was devised.

Urine specimens were received by the NEMCH Microbiology Laboratory and immediately refrigerated. Near the end of a working day, all samples were inoculated onto an Eosin Methylene Blue (EMB) blood agar plate with a 0.001-ml loop for colony counting and isolation. The original urine sample was refrigerated. By examining plates in the morning, urines with single organisms, 30,000 organisms/ml or greater could be identified. The original urine samples could then be obtained from the refrigerator and used for ATP susceptibility testing.

Experimental Plan

Samples were obtained as described previously. Forty-five ml of the urine sample were centrifuged at 8000 RCF $\times$ G for 15 minutes at 4°C. The supernate was decanted, and the tube was allowed to drain in the upright position for approximately 30 seconds. The bacterial sediment was reconstituted with 15 ml of TSB.

To establish an appropriate inoculum size, an initial ATP assay of the reconstituted sample was performed at this time. Of the 15-ml sample, 0.5 ml was subjected to ATP assay by the nonconcentrated procedure without malic acid as described earlier in this report. On the basis of the ATP level obtained, a dilution was carried out to give an A_0 ATP level of approximately 1×10^6 light units, which was shown by standard plate counts to correspond to an inoculum of approximately 1×10^6 organisms/ml. The dilution was carried out with additional TSB. Naturally, if the initial ATP assay produced less than 1×10^6 or approximately 1×10^6 , no dilution step was employed.

For example, an initial ATP level of 2.27×10^8 light units was diluted 1:100 with TSB which resulted in an A_0 of 2.05×10^6 light units; an initial ATP level of 1.14×10^7 light units was diluted 1:10 which resulted in an A_0 of 8.54×10^5 light units.

At this point, the susceptibility testing was carried out as described in the first section.

Results

The results of 24 preliminary clinical samples are given in table 5-3. The sample number, laboratory number, organism name, and number of bacteria per ml as determined by plate count are given. Light units for the A_0 and $A_{2.5}$ growth controls are given in the upper left for each sample. The far left column gives the antibiotic being tested (final concentration for each antibiotic has been listed previously). The column labeled Index gives the ATP index calculated as described previously (without the use of logs). The next column, labeled ATP Interpretation, assigns resistance (R) if the index is greater than 0.05 and sensitivity (S) if the index is less than or equal to 0.05. The column headed Laboratory Results gives results obtained from standard Kirby-Bauer agar diffusion susceptibility testing performed by the NEMCH Microbiology Laboratory. The column marked Usable indicates, by a negative sign, when a particular determination was not included in final statistical analyses. In this preliminary work, a determination was not used when the Kirby-Bauer method gave indeterminate results. The problem of correlating indeterminate results with ATP results will be considered later. A total of usable determinations for each sample is given at the bottom of this column. The last column gives an indication of when the ATP results and Kirby-Bauer results disagreed. A false-sensitive (S) means that the Kirby-Bauer result was resistant and the ATP result was sensitive. A false-resistant (R) means that the Kirby-Bauer result was sensitive and the ATP result was resistant.

Table 5-3

**Results of Preliminary Clinical Trial of the ATP Method for Determining
Antibiotic Susceptibilities Using the Nonconcentrated Procedure
without Malic Acid to Estimate Inoculum Size**

Antibiotic	Index	ATP Interpretation	Laboratory Results	Usable	Disagreement with Laboratory
Sample No. 1 Laboratory No. 160 50,000 <i>E coli</i> , <10,000 <i>coliform</i> $A_0 = 1.81 \times 10^6$ $A_{2.5} = 1.14 \times 10^8$					
Penicillin	+1.08	R	R		
Ampicillin	+1.01	R	R		
Nafcillin	+1.12	R		—	
Carbenicillin	+1.13	R		—	
Cephalothin	-0.01	S	I	—	
Tetracycline	+0.80	R	R		
Chloramphenicol	+1.16	R	R		
Clindamycin	+1.27	R	R		
Erythromycin	+0.86	R		—	
Gentamicin	-0.01	S	S		
Colistin	-0.01	S	S		
				Total = 7	
Sample No. 2 Laboratory No. 170 20-40,000 <i>Klebsiella</i> , <10,000 <i>Proteus</i> $A_0 = 1.49 \times 10^6$ $A_{2.5} = 3.75 \times 10^7$					
Penicillin	+0.96	R	R		
Ampicillin	+0.87	R	R		
Nafcillin	+0.89	R		—	
Carbenicillin	+0.64	R		—	
Cephalothin	-0.02	S	S		
Tetracycline	-0.02	S	S		
Chloramphenicol	-0.02	S	S		
Clindamycin	+0.88	R	R		
Erythromycin	+0.81	R		—	
Gentamicin	-0.03	S	S		
Colistin	-0.02	S	S		
				Total = 8	
Sample No. 3 Laboratory No. 185 >100,000 <i>E coli</i> , 10-30,000 <i>Proteus</i> $A_0 = 2.44 \times 10^6$ $A_{2.5} = 1.71 \times 10^8$					
Penicillin	+1.35	R	R		
Ampicillin	-0.01	S	S		
Nafcillin	+1.33	R		—	
Carbenicillin	-0.01	S		—	
Cephalothin	-0.01	S	S		
Tetracycline	+0.19	R	S		(false R)
Chloramphenicol	+0.02	S	S		
Clindamycin	+1.52	R	R		
Erythromycin	+1.35	R		—	
Gentamicin	-0.01	S	S		
Colistin	+0.20	R	S		
				Total = 8	(false R)
Sample No. 4 Laboratory No. 168 40,000 <i>E coli</i> , <10,000 gram negative $A_0 = 7.04 \times 10^5$ $A_{2.5} = 1.14 \times 10^8$					
Penicillin	+0.69	R	R		
Ampicillin	+0.30	R	S		(false R)
Nafcillin	+0.73	R		—	
Carbenicillin	+0.28	R		—	
Cephalothin	+0.01	S	S		
Tetracycline	+0.01	S	S		
Chloramphenicol	+0.00	S	S		
Clindamycin	+0.73	R	R		
Erythromycin	+0.56	R		—	
Gentamicin	+0.04	S	S		
Colistin	+0.03	S	S		
				Total = 8	

Table 5-3 (Continued)

Antibiotic	Index	ATP Interpretation	Laboratory Results	Usable	Disagreement with Laboratory	
Sample No. 5 Laboratory No. 593 >100,000 <i>Klebsiella</i> $A_0 = 1.66 \times 10^6$ $A_{2.5} = 1.28 \times 10^8$						
Penicillin	+1.24	R	R			
Ampicillin	+1.47	R	R			
Nafcillin	+1.55	R		—		
Carbenicillin	+1.25	R		—		
Cephalothin	-0.01	S	S			
Tetracycline	-0.00	S	S			
Chloramphenicol	-0.01	S	S			
Clindamycin	+0.98	R	R			
Erythromycin	+1.23	R		—		
Gentamicin	-0.01	S	S			
Colistin	-0.01	S	S			
				Total = 8		
Sample No. 6 Laboratory No. 674 50-100,000 <i>E. coli</i> $A_0 = 7.89 \times 10^6$ $A_{2.5} = 2.80 \times 10^8$						
Penicillin	+1.33	R	R			
Ampicillin	-0.02	S	S			
Nafcillin	+0.99	R		—		
Carbenicillin	-0.02	S		—		
Cephalothin	+0.05	S	S			
Tetracycline	+0.04	S	S			
Chloramphenicol	+0.03	S	S			
Clindamycin	+1.00	R	R			
Erythromycin	+0.95	R		—		
Gentamicin	-0.02	S	S			
Colistin	-0.03	S	S			
				Total = 8		
Sample No. 7 Laboratory No. 636 >100,000 <i>Enterococcus</i> $A_0 = 2.64 \times 10^6$ $A_{2.5} = 1.14 \times 10^8$						
Penicillin	-0.01	S	R		(false S)	
Ampicillin	-0.15	S	S			
Nafcillin	+1.18	R		—		
Carbenicillin	-0.01	S		—		
Cephalothin	+0.22	R	R			
Tetracycline	+0.74	R	S		(false R)	
Chloramphenicol	+0.03	S	S			
Clindamycin	+0.19	R	R			
Erythromycin	+0.03	S		—		
Gentamicin	+0.59	R	R			
Colistin	+1.11	R	R			
				Total = 8		
Sample No. 8 Laboratory No. 641 >100,000 <i>E. coli</i> , <10,000 <i>Enterococcus</i> $A_0 = 3.04 \times 10^6$ $A_{2.5} = 2.42 \times 10^8$						
Penicillin	+0.95	R	R			
Ampicillin	-0.01	S	S			
Nafcillin	+0.99	R		—		
Carbenicillin	-0.01	S		—		
Cephalothin	-0.01	S	S			
Tetracycline	+1.00	R	R			
Chloramphenicol	+0.01	S	S			
Clindamycin	+1.00	R	R			
Erythromycin	+0.82	R		—		
Gentamicin	-0.00	S	S			
Colistin	+0.02	S	S			
				Total = 8		

Table 5-3 (Continued)

Antibiotic	Index	ATP Interpretation	Laboratory Results	Usable	Disagreement with Laboratory
Sample No. 9 Laboratory No. 743 >100,000 <i>E coli</i> 30-50,000 <i>Klebsiella</i> $A_0 = 1.55 \times 10^6$ $A_{2.5} = 1.65 \times 10^8$					
Penicillin	+0.91	R	R		
Ampicillin	+0.00	S	S		
Nafcillin	+0.88	R		—	
Carbenicillin	-0.01	S		—	
Cephalothin	+0.01	S	S		
Tetracycline	+0.02	S	S		
Chloramphenicol	+0.01	S	S		
Clindamycin	+0.93	R	R		
Erythromycin	+0.93	R		—	
Gentamicin	-0.01	S	S		
Colistin	-0.01	S	S		
				Total = 8	
Sample No. 10 Laboratory No. 763 >100,000 <i>P mirabilis</i> $A_0 = 8.54 \times 10^5$ $A_{2.5} = 5.72 \times 10^7$					
Penicillin	+0.01	S	I	—	
Ampicillin	+0.04	S	S		
Nafcillin	+0.84	R		—	
Carbenicillin	-0.00	S		—	
Cephalothin	+0.05	S	S		
Tetracycline	+0.71	R	S		(false R)
Chloramphenicol	+0.01	S	S		
Clindamycin	+0.85	R	R		
Erythromycin	+1.00	R		—	
Gentamicin	+0.04	S	S		
Colistin	+0.73	R	R		
				Total = 7	
Sample No. 11 Laboratory No. 713 >100,000 <i>Pseudomonas</i> $A_0 = 3.08 \times 10^5$ $A_{2.5} = 7.49 \times 10^5$					
Penicillin	+0.32	R	R		
Ampicillin	+0.34	R	R		
Nafcillin	+1.00	R		—	
Carbenicillin	+0.74	R		—	
Cephalothin	+0.64	R	R		
Tetracycline	+1.36	R	R		
Chloramphenicol	+0.66	R	R		
Clindamycin	+2.77	R	R		
Erythromycin	+2.86	R		—	
Gentamicin	+0.41	R	R		
Colistin	+0.52	R	S		
				Total = 8	(false R)
Sample No. 12 Laboratory No. 211 >100,000 <i>E coli</i> <10,000 <i>Mucoid Coliform</i> $A_0 = 2.01 \times 10^6$ $A_{2.5} = 3.00 \times 10^8$					
Penicillin	+0.79	R	R		
Ampicillin	+0.00	S	S		
Nafcillin	+0.87	R		—	
Carbenicillin	+0.00	S		—	
Cephalothin	+0.03	S	S		
Tetracycline	+0.86	R	R		
Chloramphenicol	+0.01	S	S		
Clindamycin	+0.80	R		—	
Erythromycin	+0.74	R	R		
Gentamicin	-0.01	S	S		
Colistin	-0.01	S	S		
				Total = 8	

Table 5-3 (Continued)

Antibiotic	Index	ATP Interpretation	Laboratory Results	Usable	Disagreement with Laboratory
Sample No. 13 Laboratory No. 234 >100,000 <i>P mirabilis</i> $A_0 = 1.60 \times 10^7$ $A_{2.5} = 3.25 \times 10^8$					
Penicillin	-0.04	S	I	—	
Ampicillin	-0.04	S	S		
Nafcillin	+1.12	R		—	
Carbenicillin	-0.04	S		—	
Cephalothin	-0.03	S	S		
Tetracycline	+0.98	R	R		
Chloramphenicol	-0.01	S	S		
Clindamycin	+1.03	R	R		
Erythromycin	+1.06	R		—	
Gentamicin	-0.04	S	S		
Colistin	+1.05	R	R	Total = 7	
Sample No. 14 Laboratory No. 420 >100,000 <i>E coli</i> $A_0 = 1.99 \times 10^6$ $A_{2.5} = 1.96 \times 10^8$					
Penicillin	+0.98	R	R		(false R)
Ampicillin	+0.001	S	S		
Nafcillin	+1.12	R		—	
Carbenicillin	-0.01	S		—	
Cephalothin	+0.06	R	S		
Tetracycline	+0.01	S	S		
Chloramphenicol	+0.01	S	S		
Clindamycin	+1.05	R	R		
Erythromycin	+0.95	R		—	
Gentamicin	-0.01	S	S		
Colistin	-0.01	S	S	Total = 8	
Sample No. 15 Laboratory No. 412 50-100,000 <i>Klebsiella</i> $A_0 = 5.30 \times 10^5$ $A_{2.5} = 1.96 \times 10^7$					
Penicillin	+1.08	R	R		
Ampicillin	+0.40	R	R		
Nafcillin	+0.98	R		—	
Carbenicillin	+0.17	R		—	
Cephalothin	-0.00	S	S		
Tetracycline	+0.03	S	S		
Chloramphenicol	+0.03	S	S		
Clindamycin	+0.86	R	R		
Erythromycin	+0.87	R		—	
Gentamicin	-0.00	S	S		
Colistin	-0.01	S	S	Total = 8	
Sample No. 16 Laboratory No. 492 >100,000 <i>P morganii</i> $A_0 = 7.68 \times 10^5$ $A_{2.5} = 1.40 \times 10^7$					
Penicillin	+1.52	R	R		
Ampicillin	+1.10	R	R		
Nafcillin	+1.32	R		—	
Carbenicillin	-0.04	S		—	
Cephalothin	+1.47	R	R		
Tetracycline	-0.03	S	S		
Chloramphenicol	-0.03	S	S		
Clindamycin	+1.17	R	R		
Erythromycin	+1.19	R		—	
Gentamicin	-0.04	S	S		
Colistin	+1.26	R	R	Total = 8	

Table 5-3 (Continued)

Antibiotic	Index	ATP Interpretation	Laboratory Results	Usable	Disagreement with Laboratory
Sample No. 17 Laboratory No. 505 >100,000 <i>E coli</i> $A_0 = 4.74 \times 10^6$ $A_{2.5} = 1.75 \times 10^8$					
Penicillin	+1.47	R	R		
Ampicillin	-0.01	S	S		
Nafcillin	+1.29	R		—	
Carbenicillin	-0.03	S		—	
Cephalothin	+0.01	S	S		
Tetracycline	+0.02	S	S		
Chloramphenicol	+0.01	S	S		
Clindamycin	+0.77	R	R		
Erythromycin	+0.73	R		—	
Gentamicin	-0.03	S	S		
Colistin	-0.03	S	S	Total = 8	
Sample No 18 Laboratory No. 763 >100,000 <i>E coli</i> $A_0 = 4.78 \times 10^6$ $A_{2.5} = 2.61 \times 10^8$					
Penicillin	+1.14	R	R		
Ampicillin	-0.02	S	S		
Nafcillin	+0.97	R		—	
Carbenicillin	-0.02	S		—	
Cephalothin	-0.02	S	S		
Tetracycline	+0.97	R	R		
Chloramphenicol	+0.02	S	S		
Clindamycin	+0.94	R	R		
Erythromycin	+0.98	R		—	
Gentamicin	-0.02	S	S		
Colistin	-0.02	S	S	Total = 8	
Sample No. 19 Laboratory No 785 >100,000 <i>P morganii</i> $A_0 = 1.65 \times 10^6$ $A_{2.5} = 4.75 \times 10^7$					
Penicillin	+1.24	R	R		(false R)
Ampicillin	+1.26	R	S		
Nafcillin	+1.14	R		—	
Carbenicillin	-0.02	S		—	
Cephalothin	+1.21	R	R		
Tetracycline	+1.00	R	R		
Chloramphenicol	+0.15	R	R		
Clindamycin	+1.27	R	R		
Erythromycin	+1.06	R		—	
Gentamicin	-0.03	S	S		
Colistin	+0.97	R	R	Total = 8	
Sample No 20 Laboratory No. 812 >100,000 <i>Citrobacter ferundii</i> $A_0 = 2.14 \times 10^6$ $A_{2.5} = 2.10 \times 10^8$					
Penicillin	+0.77	R	R		(false S)
Ampicillin	+0.01	S	S		
Nafcillin	+0.86	R		—	
Carbenicillin	-0.01	S		—	
Cephalothin	+0.00	S	R		
Tetracycline	+0.02	S	S		
Chloramphenicol	+0.03	S	S		
Clindamycin	+0.94	R	R		
Erythromycin	+0.94	R		—	
Gentamicin	-0.01	S	S		
Colistin	-0.01	S	S	Total = 8	

Table 5-3 (Continued)

Antibiotic	Index	ATP Interpretation	Laboratory Results	Usable	Disagreement with Laboratory
Sample No. 21 Laboratory No. 881 >100,000 <i>E coli</i> $A_0 = 1.69 \times 10^6$ $A_{2.5} = 2.79 \times 10^8$					
Penicillin	+0.91	R	R		
Ampicillin	-0.00	S	S		
Nafcillin	+0.90	R		—	
Carbenicillin	-0.01	S		—	
Cephalothin	-0.00	S	S		
Tetracycline	+0.02	S	S		
Chloramphenicol	+0.02	S	S		
Clindamycin	+0.84	R	R		
Erythromycin	+0.66	R			
Gentamicin	-0.01	S	S		
Colistin	-0.01	S	S	Total = 8	
Sample No. 22 Laboratory No. 891 >100,000 <i>Candida</i> >100,000 <i>P. Morganii</i> $A_0 = 1.42 \times 10^6$ $A_{2.5} = 6.54 \times 10^7$					
Penicillin	+0.92	R	R		
Ampicillin	+0.80	R	R		
Nafcillin	+0.92	R		—	
Carbenicillin	+0.01	S		—	
Cephalothin	+0.69	R	R		
Tetracycline	+0.82	R	R		
Chloramphenicol	+0.21	R	R		
Clindamycin	+0.95	R	R		
Erythromycin	+0.82	R		—	
Gentamicin	+0.17	R	S		(false R)
Colistin	+0.89	R	R	Total = 8	
Sample No. 23 Laboratory No. 900 >100,000 <i>E coli</i> 50-100,000 <i>Enterococcus</i> $A_0 = 9.44 \times 10^6$ $A_{2.5} = 5.46 \times 10^8$					
Penicillin	+0.83	R	R		
Ampicillin	-0.00	S	S		
Nafcillin	+0.92	R		—	
Carbenicillin	-0.02	S		—	
Cephalothin	+0.01	S	S		
Tetracycline	+0.03	S	S		
Chloramphenicol	+0.01	S	S		
Clindamycin	+0.98	R	R		
Erythromycin	+0.75	R		—	
Gentamicin	-0.01	S	S		
Colistin	+0.01	S	S	Total = 8	
Sample No. 24 Laboratory No. 122 >100,000 <i>P. mirabilis</i> $A_0 = 0.06 \times 10^5$ $A_{2.5} = 1.31 \times 10^6$					
Penicillin	+0.82	R	I	—	
Ampicillin	+0.09	R	S		(false R)
Nafcillin	+0.53	R		—	
Carbenicillin	+0.00	S		—	
Cephalothin	+0.21	R	I	—	
Tetracycline	+0.33	R	R		
Chloramphenicol	+0.01	S	S		
Clindamycin	+1.31	R	R		
Erythromycin	+1.55	R		—	
Gentamicin	+0.27	R	S		(false R)
Colistin	+0.94	R	R	Total = 6	

Discussion

Twenty-four samples were run with a total of 187 usable antibiotic determinations. Overall correlation was 93.1 percent between the Kirby-Bauer method and the ATP method.

Nine samples had mixed infections; agreement for this subgroup was 94.4 percent. Three patients were on antibiotics, and agreement for this group was 87.5 percent. Patient 7 was on kanamycin, patient 11 was on gentamicin, and patient 16 was on ampicillin. Evaluation of this potential problem will require more samples for statistical significance.

Analysis of errors by individual antibiotics indicated that ampicillin had the greatest number. Three of the 12 total errors were with ampicillin. Two were with *Proteus* and one was with > 100,000 per ml *E. coli* accompanied by < 10,000 per ml of another coliform. Two errors each were encountered for cephalothin, tetracycline, gentamicin, and colistin. Penicillin had one error.

The ampicillin-*Proteus* combination difficulty had been encountered in previous work and is thought to be dependent on the mechanism of action of ampicillin. Possibly this problem could be corrected by prolongation of incubation time to 3 hours.

Conclusions

The preliminary work successfully established the following points:

- That organisms could be isolated from the urine for subsequent susceptibility testing using centrifugation
- That appropriate inocula could be established by using an initial ATP assay
- That a high correlation between the Kirby-Bauer method and the ATP method could be obtained using urine samples directly
- That samples with two organisms, in which one organism predominated, could be evaluated with ATP methods

USE OF AN ATP ASSAY (THE LONG CENTRIFUGATION METHOD) SUITABLE FOR DETECTION OF BACTERIURIAS TO ESTABLISH APPROPRIATE INOCULUM SIZE FOR SUSCEPTIBILITY TESTING

In the preliminary clinical work, an initial ATP assay was necessary to establish appropriate inoculum size for subsequent antibiotic susceptibility testing. An eventual goal of the ATP work is to first employ the ATP assay for detection of significant bacteriurias and then to apply susceptibility testing when a significant infection is found. Clearly, it would be desirable if information from the initial detection ATP assay could be used to produce appropriate inoculum sizes for subsequent susceptibility testing. Considerable time and expense could be saved if the centrifugation step required in the detection ATP assay also could be used to isolate bacteria for subsequent susceptibility tests. A method to achieve these goals was

devised. Figure 5-3, a flow chart of the method, indicates the performance of two procedures: detection of bacteriuria on the left and susceptibility testing on the right. The first centrifugation (below urine sample) serves as the first centrifugation step for the ATP assay used for detection and also serves to isolate bacteria from the urine for subsequent susceptibility testing.

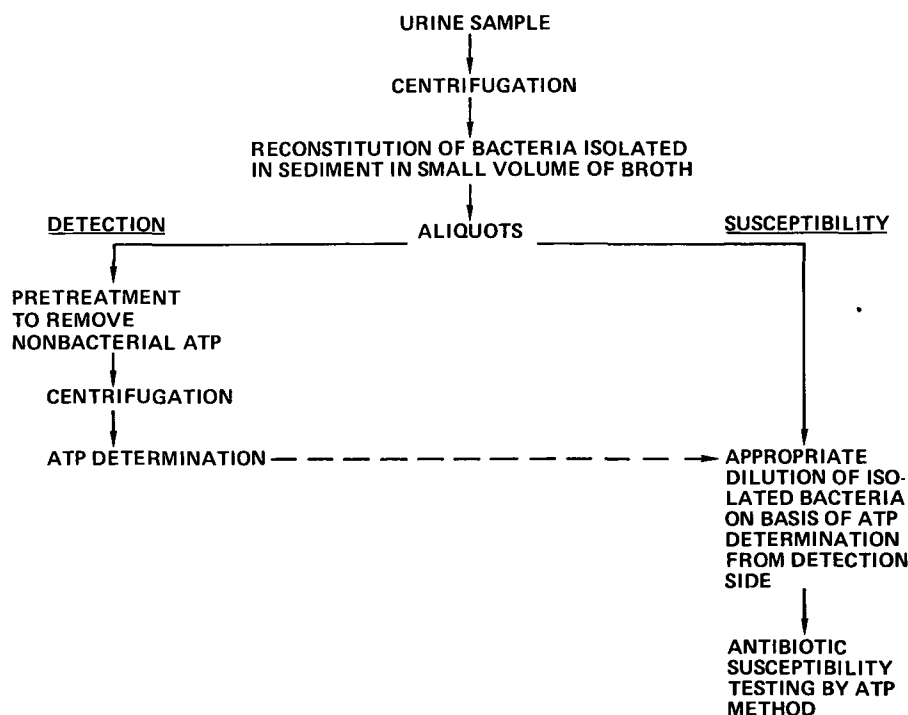


Figure 5-3 Flow chart of long centrifugation procedure for ATP assay.

After initial centrifugation, the detection assay is completed. If a significant bacteriuria is detected, susceptibility testing is carried out; if not, work on that sample is completed. The ATP level from the detection assay is used to calculate appropriate dilution of the bacterial sediment to achieve desired inoculum size.

The details of the procedures are as follows. Presently the most sensitive detection assay with best discrimination between bacterial ATP and nonbacterial ATP is the long centrifugation procedure (Chappelle et al., 1975). The detection procedure used here is the slightly modified long centrifugation procedure. In this method, 35 ml of the urine sample is centrifuged at 8000 RCF X G for 15 minutes at 4°C, and the sediment is suspended in 1.75 ml of TSB. Of this 1.75 ml, 0.5 ml is then used to complete the long centrifugation procedure. This 0.5 ml represents the sediment obtained from 10 ml of urine which is the amount called for in the long centrifugation procedure. In the established long centrifugation procedure, Triton X-100 (TX) is added prior to the initial centrifugation step. Rather than expose bacteria that will be used in subsequent susceptibility testing to TX, we have chosen to add

the TX along with the apyrase after the initial centrifugation. This should have an equivalent effect at this point in the assay. The remainder of the assay is performed according to the established technique. The exact manipulations are given in Procedures 1 and 2 in Appendix B.

Establishing Appropriate Inoculum for Susceptibility Testing

The ATP level obtained from the detection assay was then used to establish an appropriate inoculum size for susceptibility testing.

Based on the ATP level obtained, a certain dilution was carried out. An inoculum size of at least 1×10^5 organisms/ml, but not much greater than 1×10^6 organisms/ml, was needed for susceptibility testing. Previous work indicated that these inoculum sizes corresponded to about 1×10^5 to 1×10^7 light units for the A_0 's by the nonconcentrated procedure without malic acid. A dilution schedule to achieve this range of A_0 based on the results from the long centrifugation procedure was established. The details of how this dilution schedule was established are given in Experiment 4 in Appendix B. The dilution schedule is given in table 5-4.

Table 5-4
Dilution Schedule to Achieve Desired Inoculum Size

Long Centrifugation ATP Levels (light units)	Desired Change from the Original Bacterial Level in the Urine	Actual Manipulation of 1.25 ml Aliquot (which already represents a 20-fold concentration of the original urine)
1.0×10^6	No significant bacteria	Stop procedure
$1.0 \times 10^6 - 1.0 \times 10^8$	3-fold concentration	6-fold dilution
$1.0 \times 10^8 - 3.0 \times 10^8$	No change from original level	20-fold dilution
$3.0 \times 10^8 - 3.0 \times 10^9$	10-fold dilution	200-fold dilution
3.0×10^9	100-fold dilution	2000-fold dilution

The A_0 's achieved with this dilution schedule are given in the results of the further clinical trials. The great majority of A_0 's were in the desired 1×10^5 to 1×10^7 light unit range.

ADDITION OF KANAMYCIN AND STREPTOMYCIN TO ANTIBIOTICS BEING EVALUATED

The Microbiology Laboratory at NEMCH includes kanamycin and streptomycin in antibiotic susceptibility testing for urinary pathogens. Kirby-Bauer results would be available from the Microbiology Laboratory for each sample. Therefore, it was desirable to include these antibiotics in further clinical trials of the ATP method.

An approach similar to that employed by Dr. Vellend (described in Section 4 of this document) was used. An appropriate antibiotic concentration was sought first. Concentrations that represented the MIC breakpoint for resistant and susceptible strains, and a concentration midway between these values were evaluated against the previously employed reference strains. Using these results, the following concentrations were chosen for additional evaluation: 15 $\mu\text{g/ml}$ of kanamycin and 10 $\mu\text{g/ml}$ of streptomycin.

Details of the experiments used to arrive at these values are given in Experiment 3 in Appendix B. Additional evaluation of various strains would take place as part of the clinical trial. The correlation achieved for these antibiotics with Kirby-Bauer results would indicate the appropriateness of these concentrations.

MODIFICATION OF CERTAIN TECHNICAL ASPECTS OF THE METHODS TO INCREASE EFFICIENCY AND ACCURACY

Antibiotics, when originally made up at 2-week intervals, were diluted to appropriate concentrations with sterile distilled water, aliquoted into individual polypropylene tubes that were suitable for ATP assay, and frozen. On the day of use, it was a matter of selecting appropriate previously aliquoted tubes rather than pipetting individual aliquots each day.

Rather than use 0.1 ml of antibiotic solution and 0.9 ml of broth with bacterial inoculum, 0.05 ml of antibiotic solution (or distilled water for growth control) and 0.45 ml of broth were chosen. This change would allow the assay to be performed by addition of reagents, without having to withdraw 0.5 ml into a separate tube. The modification represents a considerable savings of time and equipment (pipettes and polypropylene tubes), and probably improves accuracy.

Rather than prepare luciferase twice and perform separate ATP assays for A_0 's, the samples were taken through the addition of apyrase, nitric acid, and distilled water, and then stored at room temperature until $A_{2.5}$ and B_{1-12} were ready for assaying. All samples were then assayed using the same luciferase preparation. Experiments to confirm the stability of the extracted and diluted bacterial ATP in the A_0 were performed. The details are given in Experiments 1 and 2 in Appendix B. The ATP levels did not change significantly after even 5 hours for any of the reference organisms tested. This modification conserved luciferase and perhaps increased accuracy by employing the same luciferase for corresponding A_0 's, $A_{2.5}$'s, and B's.

FURTHER EVALUATION OF CLINICAL URINE SAMPLES AND DETAILED ANALYSIS OF ERRORS

This evaluation of clinical urine samples employed the changes discussed above: the use of the long centrifugation method, the addition of kanamycin and streptomycin, and other modifications. The exact manipulations of samples are given in Procedures 1 and 2 in Appendix B. Samples were selected in the manner described in Section 4.

Results

Table 5-5 gives the results of 42 clinical specimens. The format is similar to that used in tables 5-2 and 5-3.

Particular caution was exercised in recording Kirby-Bauer data. The column labeled KB gives measurements of zones of inhibition obtained from standard Kirby-Bauer agar diffusion susceptibility testing as performed by the NEMCH Microbiology Laboratory. The column labeled KB Interpretation gives the interpretations of the Kirby-Bauer results based on measured disc diameters. In table 5-6, the standard criteria used for assigning Resistant or Sensitive on a basis of zone diameters are given. Diameters falling between critical values for resistance and sensitivity are considered indeterminate. The column labeled Lab Results is the Microbiology Laboratory interpretation of the Kirby-Bauer zones. This is based on an estimate of zone sizes and not on precise measurements as we took them. In difficult cases where zone sizes were near the cutoff points, errors could certainly arise because of such estimates. In these cases, our measured results were accepted as correct.

In the column labeled Usable, a negative sign indicates when a particular determination was not used in calculations. When the Kirby-Bauer method gave indeterminate results, the point was not included in initial analyses of the data. If the Kirby-Bauer method performed by the NEMCH Microbiology Laboratory disagreed with the Kirby-Bauer method performed by us (indicated by an asterisk) the point was generally not used. However, if our Kirby-Bauer interpretation agreed with the ATP interpretation, the point was accepted. The usable total for each sample is given at the bottom of this column. The last column, Disagreement, indicates disagreement between the ATP interpretation and the Kirby-Bauer interpretation. Kirby-Bauer results are accepted as correct. Therefore, if the Kirby-Bauer result was sensitive and ATP result was resistant, this was called false-resistant (false R). If the Kirby-Bauer result was resistant and the ATP result was sensitive, this was called false-sensitive (false S).

Discussion of Results

Considering the entire group of 42 samples, the correlation between Kirby-Bauer results and ATP results was 92.5 percent. Of the 42 samples, 7 were mixed infections as indicated on the individual charts in table 5-5. Agreement for the 35 nonmixed samples was 92.9 percent with 365 total determinations and 26 errors.

It is best to consider the nonmixed and mixed samples separately in an attempt to analyze sources of errors. Mixed infections here gave a similar degree of correlation; nonetheless, the presence of more than one organism could introduce an intrinsic source of error.

Table 5-5

**Results of Clinical Trial of Final Proposed ATP Method for Determining
Antibiotic Susceptibilities Using Long Centrifugation Procedure for
Establishing Inoculum Size and Testing Additional Antibiotics**

Antibiotic	Index	ATP Interpretation	KB	K-B Interpretation	Laboratory Results	Disagreement
Sample No 967 $A_0 = 3.60 \times 10^6$, $A_{2.5} = 2.23 \times 10^5$ >100,000 <i>P. mirabilis</i> , <10,000 coliform						
Penicillin	+0.22	R	16	R	I	1 (false R)
Ampicillin	+0.12	R	26	S	S	
Nafcillin	+0.69	R	0	R		
Carbencillin	+0.31	R	29			
Cephalothin	-0.01	S	22	S	S	
Tetracycline	+0.40	R	0	R	R	
Chloramphenicol	+0.00	S	23	S	S	
Clindamycin	+0.76	R	0	R	R	
Erythromycin	+0.89	R	0	R		
Gentamicin	-0.01	S				
Colistin	+0.42	R	0	R	R	
Kanamycin					S	
Streptomycin					S	
Nitrofurantoin	+0.12	R	10.0	R	R	
					Usable Total = 10	
Sample No 946 $A_0 = 1.90 \times 10^6$, $A_{2.5} = 2.30 \times 10^8$ >100,000 <i>K. pneumoniae</i>						
Penicillin	+0.57	R	10	R	R	1 (false R)
Ampicillin	+0.18	R	19	S	S	
Nafcillin	+0.78	R	0	R		
Carbencillin	+0.76	R	21			
Cephalothin	-0.01	S	27	S	S	
Tetracycline	+0.02	S	23	S	S	
Chloramphenicol	+0.01	S	30	S	S	
Clindamycin	+0.83	R	0	R	R	
Erythromycin	+0.79	R	13	R		
Gentamicin	-0.01	S		S	S	
Colistin	+0.01	S	16	S	S	
Kanamycin					S	
Streptomycin					S	
Nitrofurantoin	+0.01	S	23	S	S	
					Usable Total = 11	
Sample No. 040 $A_0 = 3.49 \times 10^5$, $A_{2.5} = 2.10 \times 10^8$ >100,000 <i>Klebsiella</i> , <10,000 <i>Proteus</i>						
Penicillin	+1.00	R	0	R	R	
Ampicillin	+1.00	R	10	R	R	
Nafcillin	+0.93	R	0	R		
Carbencillin	+0.97	R	10			
Cephalothin	-0.00	S	22	S	S	
Tetracycline	+0.01	S	20	S	S	
Chloramphenicol	+0.01	S	25	S	S	
Clindamycin	+0.91	R	0	R	R	
Erythromycin	+0.86	R	10	R		
Gentamicin	-0.00	S				
Colistin	-0.00	S	15	S	S	
Kanamycin					S	
Streptomycin					S	
Nitrofurantoin	+0.00	S	18	S	S	
					Usable Total = 10	

Table 5-5 (Continued)

Antibiotic	Index	ATP Interpretation	KB	K-B Interpretation	Laboratory Results	Disagreement
Sample No. 10 $A_0 = 5.30 \times 10^5$, $A_{2.5} = 5.77 \times 10^6$ >100,000 <i>Pseudomonas</i>						
Penicillin	+0.96	R	0	R	R	
Ampicillin	+1.07	R	0	R	R	
Nafcillin	+1.06	R	0	R		
Carbencillin	+0.59	R				
Cephalothin	+0.70	R	0	R	R	
Tetracycline	+0.31	R	0	R	R	
Chloramphenicol	+0.78	R	0	R	R	
Clindamycin	+1.25	R	0	R	R	
Erythromycin	+0.74	R	0	R		
Gentamicin	-0.05	S				
Colistin	-0.06	S	15	S	S	
Kanamycin					R	
Streptomycin					I	
Nitrofurantoin	+0.75	R	0	R	R	
					Usable Total = 10	
Sample No. 200 $A_0 = 4.50 \times 10^5$, $A_{2.5} = 5.04 \times 10^7$ 30,000 <i>E coli</i>						
Penicillin	+0.93	R	0	R	R	
Ampicillin	+0.00	S	18	S	S	
Nafcillin	+0.85	R	0	R		
Carbencillin	+0.01	S	26	S		
Cephalothin	+0.00	S	21	S	S	
Tetracycline	+0.02	S	19	S	S	
Chloramphenicol	+0.02	S	26	S	S	
Clindamycin	+0.82	R	0	R	R	
Erythromycin	+0.66	R	13	R		
Gentamicin	+0.00	S				
Colistin	+0.00	S	14	S	S	
Kanamycin					S	
Streptomycin					S	
Nitrofurantoin	+0.002	S	22	S	S	
Sample No. 229 $A_0 = 1.60 \times 10^6$, $A_{2.5} = 3.01 \times 10^7$ 50 - 100,000 <i>P mirabilis</i>						
Penicillin	+0.00	S	16	I	I	
Ampicillin	-0.01	S	24	S	S	
Nafcillin	+1.12	R	0	R		
Carbencillin	+0.01	S	28			
Cephalothin	+0.01	S	22	S	S	
Tetracycline	+1.69	R	0	R	R	
Chloramphenicol	+0.02	S	30	S	S	
Clindamycin	+1.14	R	0	R	R	
Erythromycin	+0.99	R	0	R		
Gentamicin	+0.01	S				
Colistin	+0.84	R	0	R	R	
Kanamycin					S	
Streptomycin					S	
Nitrofurantoin	+0.11	R	10	R	R	
					Usable Total = 9	

Table 5-5 (Continued)

Antibiotic	Index	ATP Interpretation	KB	K-B Interpretation	Laboratory Results	Disagreement
Sample No. 363, $A_0 = 5.07 \times 10^5$, $A_{2.5} = 1.37 \times 10^7$ >100,000 <i>P rettgeri</i>						
Penicillin	+0.69	R	0	R	R	(false R)
Ampicillin	+0.29	R	19.5	S	S	
Nafcillin	+1.05	R	0	R		
Carbencillin	-0.02	S				(false R)
Cephalothin	+0.56	R	20	S	S	
Tetracycline	+1.06	R	0	R	R	
Chloramphenicol	+0.03	S	9	R	R	
Clindamycin	+0.98	R	0	R	R	
Erythromycin	+1.32	R	0	R		
Gentamicin	-0.01	S		S	S	
Colistin	+0.92	R	0	R	R	
Kanamycin					S	
Streptomycin					S	
Nitrofurantoin	+0.95	R	0	R	R	
					Usable Total = 11	
Sample No. 361, $A_0 = 3.63 \times 10^5$, $A_{2.5} = 2.57 \times 10^7$ >100,000 <i>E coli</i>						
Penicillin	+0.98	R		R	R	(false R)
Ampicillin	+0.07	R		S	S	
Nafcillin	+1.00	R				
Carbencillin	+0.12	R				(false R)
Cephalothin	+0.10	R		S	S	
Tetracycline	+0.02	S		S	S	
Chloramphenicol	+0.006	S		S	S	
Clindamycin	+0.96	R		R	R	
Erythromycin	+0.67	R				
Gentamicin	-0.00	S		S	S	
Colistin	-0.01	S		S	S	
Kanamycin				S	S	
Streptomycin				S	S	
Nitrofurantoin	-0.005	S		S	S	
					Usable Total = 9	
Sample No. 584, $A_0 = 6.53 \times 10^5$, $A_{2.5} = 2.55 \times 10^6$ >100,000 <i>Proteus</i>						
Penicillin	+3.57	R	0	R	R	
Ampicillin	+2.80	R	0	R	R	
Nafcillin	+3.06	R	0	R		
Carbencillin						
Cephalothin	+3.23	R	13	R	R	
Tetracycline	+3.36	R	0	R	R	
Chloramphenicol	+0.88	R	8	R	R	
Clindamycin	+3.68	R	0	R	R	
Erythromycin	+2.36	R	0	R		
Gentamicin	+1.90	R	12	R	R	
Colistin	+2.99	R	0	R	R	
Kanamycin	+3.47	R	0	R	R	
Streptomycin	+1.52	R	12	I	I	
Nitrofurantoin	+0.17	R	0	R	R	
					Usable Total = 12	

Table 5-5 (Continued)

Antibiotic	Index	ATP Interpretation	KB	K-B Interpretation	Laboratory Results	Disagreement
Sample No 640, $A_0 = 6.96 \times 10^5$, $A_{2.5} = 1.83 \times 10^6$ 50,000 - 100,000 <i>E coli</i> , <10,000 Coliform						
Penicillin	+0.92	R	0	R	R	
Ampicillin	+0.84	R	0	R	R	
Nafcillin	+1.00	R	0	R		
Cephalothin	-0.01	S	21	S	S	
Tetracycline	+0.79	R	0	R	R	
Chloramphenicol	-0.03	S	21	S	S	
Clindamycin	+0.75	R	0	R	R	
Erythromycin	+0.65	R	12	R		
Gentamicin	-0.09	S	26	S	S	
Colistin	-0.28	S	17	S	S	
Kanamycin	-0.12	S	22.5	S	S	
Streptomycin	-0.09	S	16	S	S	
Nitrofurantoin	-0.28	S	20	S	S	
Usable Total = 13						
Sample No 650; $A_0 = 3.26 \times 10^6$, $A_{2.5} = 1.42 \times 10^8$ >100,000 <i>E coli</i>						
Penicillin	+1.00	R	10	R	R	
Ampicillin	+0.03	S	22	S	S	
Nafcillin	+0.94	R	0	R		
Cephalothin	-0.01	S	22	S	S	
Tetracycline	+0.02	S	21	S	S	
Chloramphenicol	+0.01	S	27	S	S	
Clindamycin	+2.22	R	0	R	R	
Erythromycin			15.5	I		
Gentamicin			24	S	S	
Colistin	+0.07	R	15	S	S	(false R)
Kanamycin	-0.02	S	22	S	S	
Streptomycin	-0.02	S	16	S	S	
Nitrofurantoin	-0.01	S	23	S	S	
Usable Total = 11						
Sample No. 739, $A_0 = 1.34 \times 10^6$, $A_{2.5} = 9.54 \times 10^7$ 30,000 - 50,000 <i>E coli</i> , <10,000 <i>Proteus</i> and <i>Enterococcus</i>						
Penicillin	+1.04	R	0	O	R	
Ampicillin	-0.01	S	13.5*	I	S	
Nafcillin	+0.84	R	0	R		
Cephalothin	+0.01	S	7*	R	I	(false S)
Tetracycline	+0.02	S	0	R	R	(false S)
Chloramphenicol	+0.01	S	23	S	R	
Clindamycin	+0.99	R	0	R	R	
Erythromycin	+0.45	R	0	R		
Gentamicin	-0.01	S	14	S	R	
Colistin	+0.01	S	0, 16*	S	R	
Kanamycin	+0.01	S	18	S	S	
Streptomycin	+0.07	R	13.5	I	S	
Nitrofurantoin	-0.01	S	8	R	R	(false S)
Usable Total = 10						

* Disagreement

Table 5-5 (Continued)

Antibiotic	Index	ATP Interpretation	KB	K-B Interpretation	Laboratory Results	Disagreement
Sample No 749, $A_0 = 1.93 \times 10^6$, $A_{2.5} = 7.16 \times 10^7$ >100,000 <i>P. rettgeri</i>						
Penicillin	+0.71	R	10	R	R	(false R)
Ampicillin	+0.16	R	22	S	S	
Nafcillin	+0.90	R	0	R		
Cephalothin	+0.42	R	22	S	S	
Tetracycline	+0.92	R	0	R	R	
Chloramphenicol	+0.12	R	12	R	R	
Clindamycin	+0.98	R	0	R	R	
Erythromycin	+1.03	R	0	R		
Gentamycin	-0.00	S	19	S	S	
Colistin	+0.87	R	0	R	R	
Kanamycin	-0.01	S	25	S	S	
Streptomycin	-0.00	S	18	S	S	
Nitrofurantoin	+0.84	R		R	R	
Usable Total = 13						
Sample No 978, $A_0 = 2.77 \times 10^5$, $A_{2.5} = 7.25 \times 10^6$ 50 - 100,000 <i>E. coli</i> , <10,000 Diphtheroids						
Penicillin	+1.03	R	0	R	R	
Ampicillin	+0.92	R	0	R	R	
Nafcillin	+0.87	R	0	R		
Cephalothin	+0.07	R	17	I	I	
Tetracycline	+0.65	R	0	R	R	
Chloramphenicol	+0.03	S	26	S	S	
Clindamycin	+0.59	R	0	R	R	
Erythromycin	+0.48	R	13	R		
Gentamicin	-0.02	S	24	S	S	
Colistin	-0.03	S	15	S	S	
Kanamycin	+0.66	R	0	R	R	
Streptomycin	+0.73	R	0	R	R	
Nitrofurantoin	-0.02	S	23	S	S	
Usable Total = 13						
Sample No 994, $A_0 = 9.14 \times 10^5$, $A_{2.5} = 3.61 \times 10^7$ >100,000 <i>E. coli</i>						
Penicillin	+0.99	R	0	R	R	
Ampicillin	-0.02	S	18.5	S	S	
Nafcillin	+1.15	R	0	R		
Cephalothin	-0.02	S	21	S	S	
Tetracycline	+0.01	S	21	S	S	
Chloramphenicol	+0.01	S	28	S	S	
Clindamycin	+0.94	R	0	R	R	
Erythromycin	+0.69	R	13	R		
Gentamicin	-0.02	S	25.5	S	S	
Colistin	-0.02	S	16	S	S	
Kanamycin	-0.02	S	23	S	S	
Streptomycin	+0.04	S	18	S	S	
Nitrofurantoin	-0.02	S	20	S	S	
Usable Total = 13						

Table 5-5 (Continued)

Antibiotic	Index	ATP Interpretation	KB	K-B Interpretation	Laboratory Results	Disagreement
Sample No. 997, $A_0 = 3.99 \times 10^5$, $A_{2.5} = 2.12 \times 10^7$ >100,000 <i>E. coli</i>						
Penicillin	+0.96	R	0	R	R	
Ampicillin	+0.91	R	0	R	R	
Nafcillin	+0.92	R	0	R		
Cephalothin	-0.01	S	19	S	S	
Tetracycline	+0.80	R	0	R	R	
Chloramphenicol	+0.02	S	24	S	S	
Clindamycin	+0.88	R	0	R	R	
Erythromycin	+0.83	R	10	R		
Gentamicin	-0.01	S	27	S	S	
Colistin	-0.01	S	16.5	S	S	
Kanamycin	+0.59	R	0	R	R	
Streptomycin	+0.88	R	0	R	R	
Nitrofurantoin	-0.01	S	22	S	S	
Usable Total = 13						
Sample No. 168, $A_0 = 2.72 \times 10^6$, $A_{2.5} = 1.96 \times 10^8$ >100,000 <i>E. coli</i> , 30 - 50,000 <i>P. mirabilis</i> (<i>E. coli</i>) (<i>Proteus</i>)						
			<i>E. coli</i>			
Penicillin	+0.98	R	0	R	R	I
Ampicillin	+1.25	R	0	R	R	S
Nafcillin	+1.06	R	0	R		
Cephalothin	-0.00	S	15	I	S	S
Tetracycline	+0.82	R	0	R	R	R
Chloramphenicol	-0.01	S	27	S	S	S
Clindamycin	+1.02	R	0	R	R	R
Erythromycin	+0.88	R	<18	I		
Gentamicin	-0.01	S	25	S	S	S
Colistin	+0.01	S	16	S	S	R
Kanamycin	-0.01	S	22	S	S	S
Streptomycin	+1.09	R	9	R	R	S
Nitrofurantoin	-0.00	S	23	S	S	R
Usable Total = 11						
Sample No. 191, $A_0 = 4.63 \times 10^6$, $A_{2.5} = 6.69 \times 10^7$ >100,000 <i>E. coli</i>						
Penicillin	+1.47	R	8	R	R	
Ampicillin	+0.26	R	22	S	S	(false R)
Nafcillin	+1.48	R	0	R		
Cephalothin	+0.20	R	23	S	S	(false R)
Tetracycline	-0.01	S	21	S	S	
Chloramphenicol	-0.01	S	25	S	S	
Clindamycin	+1.17	R	0	R	R	
Erythromycin	+0.88	R	16	I		
Gentamicin	-0.07	S	22	S	S	
Colistin	-0.07	S	17	S	S	
Kanamycin	-0.03	S	18.5	S	S	
Streptomycin	+0.16	R	17	S	I	(false R)
Nitrofurantoin	-0.03	S	27	S	S	
Usable Total = 12						

Table 5-5 (Continued)

Antibiotic	Index	ATP Interpretation	KB	K-B Interpretation	Laboratory Results	Disagreement
Sample No. 232, $A_0 = 8.58 \times 10^6$, $A_{2.5} = 3.34 \times 10^8$ >100,000 <i>Klebsiella</i>						
Penicillin	+0.82	R	0	R	R	
Ampicillin	+0.78	R	9	R	R	
Nafcillin	+0.93	R	0	R		
Cephalothin	-0.03	S	22	S	S	
Tetracycline	+0.02	S	17	I	I	
Chloramphenicol	+0.02	S	26	S	S	
Clindamycin	+0.70	R	0	R	R	
Erythromycin	+0.39	R	12	R		
Gentamicin	-0.03	S	25	S	S	
Colistin	-0.03	S	16	S	S	
Kanamycin	-0.02	S	23	S	I	
Streptomycin	-0.01	S	21	S	I	
Nitrofurantoin	-0.01	S	22	S		
Usable Total = 12						
Sample No. 256, $A_0 = 6.63 \times 10^6$, $A_{2.5} = 2.44 \times 10^8$ >100,000 <i>Serratia marcescens</i>						
Penicillin	+1.05	R	0	R	R	
Ampicillin	+1.09	R	0	R	R	
Nafcillin	+0.86	R	0	R		
Cephalothin	+0.76	R	0	R	R	
Tetracycline	+0.80	R	0	R	R	
Chloramphenicol	+0.06	R	13*	I	R	
Clindamycin	+0.76	R	0	R	R	
Erythromycin	+0.87	R	9	R		
Gentamicin	+0.70	R	9	R	R	
Colistin	+0.49	R	0	R	R	
Kanamycin	+0.84	R	0	R	R	
Streptomycin	+0.76	R	0	R	R	
Nitrofurantoin	+0.47	R	10	R	R	
Usable Total = 12						
Sample No. 294, $A_0 = 5.94 \times 10^6$, $A_{2.5} = 4.36 \times 10^8$ >100,000 <i>Klebsiella</i>						
Penicillin	+0.95	R	0	R	R	
Ampicillin	+0.78	R	10	R	R	
Nafcillin	+0.99	R	0	R		
Cephalothin	-0.01	S	26	S	S	
Tetracycline	+0.03	S	21	S	S	
Chloramphenicol	+0.04	S	30	S	S	
Clindamycin	+0.97	R	0	R	R	
Erythromycin	+0.88	R	12.5	R		
Gentamicin	-0.01	S	29	S	S	
Colistin	-0.01	S	0*	R	S	
Kanamycin	-0.01	S	26	S	S	
Streptomycin	-0.01	S	17	S	S	
Nitrofurantoin	+0.13	R	19	S		(false R)
Usable Total = 12						

* Disagreement

Table 5-5 (Continued)

Antibiotic	Index	ATP Interpretation	KB	K-B Interpretation	Laboratory Results	Disagreement
Sample No. 466, $A_0 = 5.73 \times 10^6$, $A_{2.5} = 2.16 \times 10^8$ >100,000 <i>E coli</i>						
Penicillin	+1.15	R	0	R	R	(false R)
Ampicillin	-0.01	S	19	S	S	
Nafcillin	+0.92	R	0	R		
Cephalothin	-0.00	S	22	S	S	
Tetracycline	+1.11	R	8	R	R	
Chloramphenicol	+0.03	S	27	S	S	
Clindamycin	+1.19	R	0	R	R	
Erythromycin	+1.10	R	12.5	R		
Gentamicin	+0.08	R	27	S	S	
Colistin	-0.03	S	16	S	S	
Kanamycin	+0.14	R	24*	S	R	
Streptomycin	+1.17	R	0	R	R	
Nitrofurantoin	+0.82	R	14	R	R	
Usable Total = 12						
Sample No 504, $A_0 = 3.69 \times 10^6$, $A_{2.5} = 2.82 \times 10^8$ >100,000 <i>E coli</i>						
Penicillin	+0.90	R	7	R	R	
Ampicillin	-0.01	S	20	S	S	
Nafcillin	+0.94	R	0	R		
Cephalothin	-0.01	S	23	S	S	
Tetracycline	+0.85	R	0	R	R	
Chloramphenicol	+0.00	S	28	S	S	
Clindamycin	+0.94	R	0	R	R	
Erythromycin	+0.84	R	15	R		
Gentamicin	-0.01	S	27	S	S	
Colistin	-0.01	S	17	S	S	
Kanamycin	-0.01	S	24	S	S	
Streptomycin	-0.01	S	21	S	S	
Nitrofurantoin	-0.01	S	23	S	S	
Usable Total = 13						
Sample No. 512, $A_0 = 3.88 \times 10^6$, $A_{2.5} = 1.33 \times 10^8$ >100,000 <i>E coli</i>						
Penicillin	+0.98	R	0	R	R	(false R)
Ampicillin	-0.01	S	14	S	S	
Nafcillin	+0.88	R	0	R		
Cephalothin	+0.14	R	21	S	S	
Tetracycline	+0.94	R	0	R	R	
Chloramphenicol	+0.03	S	28	S	S	
Clindamycin	+0.90	R	0	R	R	
Erythromycin	+0.98	R	15	I		
Gentamicin	-0.02	S	30	S	S	
Colistin	-0.03	S	18	S	S	
Kanamycin	-0.02	S	29	S	S	
Streptomycin	+0.59	R	0	R	R	
Nitrofurantoin	-0.01	S	24	S	S	
Usable Total = 12						

* Disagreement

Table 5-5 (Continued)

Antibiotic	Index	ATP Interpretation	KB	K-B Interpretation	Laboratory Results	Disagreement
Sample No. 524, $A_0 = 1.36 \times 10^7$, $A_{2.5} = 2.19 \times 10^8$ >100,000 <i>E. coli</i>						
Penicillin	+0.99	R	0	R	R	
Ampicillin	+0.94	R	20	S	S	(false R)
Nafcillin	-0.05	S	0	R		(false S)
Cephalothin	+0.10	R	25	S	S	(false R)
Tetracycline	+0.01	S	25	S	S	
Chloramphenicol	+0.07	R	28	S	S	(false R)
Clindamycin	+0.88	R	0	R	R	
Erythromycin	+0.86	R	15	I		
Gentamicin	-0.06	S	28	S		
Colistin	-0.06	S	18	S	S	
Kanamycin	-0.05	S	26	S	S	
Streptomycin	-0.00	S	22	S	S	
Nitrofurantoin	-0.02	S	24	S	S	
Usable Total = 12						
Sample No. 751, $A_0 = 9.87 \times 10^5$, $A_{2.5} = 4.32 \times 10^7$ >100,000 <i>Klebsiella</i>						
Penicillin	+1.47	R	0	R	R	
Ampicillin	+1.56	R	10.5	R	R	
Nafcillin	+1.48	R	0	R		
Cephalothin	-0.01	S	25	S	S	
Tetracycline	+0.04	S	15	I	R	
Chloramphenicol	-0.01	S	28	S	S	
Clindamycin	+1.52	R	0	R	R	
Erythromycin	+1.08	R	12	R		
Gentamicin	-0.01	S	28	S	S	
Colistin	-0.02	S	17	S	S	
Kanamycin	+0.02	S	25	S	S	
Streptomycin	+0.58	R	10	R	R	
Nitrofurantoin	+0.13	R	18	S	S	(false R)
Usable Total = 12						
Sample No. 774, $A_0 = 8.57 \times 10^5$, $A_{2.5} = 3.43 \times 10^7$ 50 - 100,000 <i>P. mirabilis</i>						
Penicillin	-0.01	S	19	I	I	
Ampicillin	-0.01	S	26	S	S	
Nafcillin	+1.01	R	0	R		
Cephalothin	+0.00	S	23	S		
Tetracycline	+0.84	R	75	R	R	
Chloramphenicol	-0.00	S	30	S	S	
Clindamycin	+1.02	R	0	R	R	
Erythromycin	+1.18	R	0	R		
Gentamicin	-0.01	S	26	S	S	
Colistin	+0.75	R	0	R	R	
Kanamycin	-0.01	S	22	S	S	
Streptomycin	+0.07	R	20*	S	S	(false R)
Nitrofurantoin	+0.36	R	9	R	R	
Usable Total = 12						

* Disagreement

Table 5-5 (Continued)

Antibiotic	Index	ATP Interpretation	KB	K-B Interpretation	Laboratory Results	Disagreement
Sample No 795, $A_0 = 9.48 \times 10^6$, $A_{2.5} = 3.77 \times 10^8$ >100,000 <i>E. coli</i>						
Penicillin	+0.92	R	0	R	R	
Ampicillin	-0.01	S	19	S	S	
Nafcillin	+0.88	R	0	R		
Cephalothin	-0.00	S	22	S	S	
Tetracycline	+0.02	S	25	S	S	
Chloramphenicol	+0.02	S	28	S	S	
Clindamycin	+0.98	R	0	R	R	
Erythromycin	+0.92	R	12.5	R		
Gentamicin	-0.02	S	28	S	S	
Colistin	-0.03	S	18	S	S	
Kanamycin	-0.02	S	25	S	S	
Streptomycin	+0.02	S	21	S	I	
Nitrofurantoin	+0.01	S	23	S	S	
Usable Total = 13						
Sample No 817, $A_0 = 2.30 \times 10^7$, $A_{2.5} = 5.00 \times 10^8$ >100,000 <i>E. coli</i>						
Penicillin	+1.04	R	0	R	R	
Ampicillin	-0.02	S	22	S	S	
Nafcillin	+0.87	R	0	R		
Cephalothin	-0.03	S	26	S	S	
Tetracycline	+0.04	S	23	S	S	
Chloramphenicol	+0.03	S	27	S	S	
Clindamycin	+1.01	R	0	R	R	
Erythromycin	+0.91	R	15	I		
Gentamicin	-0.04	S	28	S	S	
Colistin	-0.05	S	18	S	S	
Kanamycin	-0.03	S	26	S	S	
Streptomycin	+0.02	S	20*	S	I	
Nitrofurantoin	-0.02	S	23	S	S	
Usable Total = 12						
Sample No 837, $A_0 = 5.76 \times 10^6$, $A_{2.5} = 5.58 \times 10^7$ >100,000 <i>Staphylococcus coagulans megali</i>						
Penicillin	-0.08	S	28*	S	R	
Ampicillin	-0.08	S	30*	S	R	
Nafcillin	-0.10	S	26	S		
Cephalothin	-0.09	S	42	S	S	
Tetracycline	-0.01	S	25	S	S	
Chloramphenicol	+0.04	S	30	S	S	
Clindamycin	+0.03	S	30	S	S	
Erythromycin	+0.05	S	40	S		
Gentamicin	-0.03	S	39	S	S	
Colistin	+1.20	R	0	R	R	
Kanamycin	-0.00	S	32	S	S	
Streptomycin	-0.07	S	24	S	I	
Nitrofurantoin	-0.02	S	29	S	S	
Usable Total = 13						

* Disagreement

Table 5-5 (Continued)

Antibiotic	Index	ATP Interpretation	KB	K-B Interpretation	Laboratory Results	Disagreement
Sample No 860, $A_0 = 4.49 \times 10^6$, $A_{2.5} = 3.35 \times 10^8$ >100,000 <i>Klebsiella</i>						
Penicillin	+0 98	R	0	R	R	(false R)
Ampicillin	+1.12	R	0	R	R	
Nafcillin	+0 96	R	0	R		
Cephalothin	-0 00	S	16*	I	S	
Tetracycline	+0 01	S	18*	I	S	
Chloramphenicol	+0 01	S	23	S	S	
Clindamycin	+0.99	R	0	R	R	
Erythromycin	+0 95	R	8	R		
Gentamicin	-0 01	S	28	S	S	
Colistin	-0.01	S	15	S	S	
Kanamycin	-0 01	S	27	S	S	
Streptomycin	+1.00	R	0	R	R	
Nitrofurantoin	+0 34	R	18	S	S	
Usable Total = 11						
Sample No 883, $A_0 = 4.23 \times 10^6$, $A_{2.5} = 2.69 \times 10^8$ >100,000 <i>E coli</i>						
Penicillin	+1.01	R	0	R	R	
Ampicillin	-0 01	S	22	S	S	
Nafcillin	+1.03	R	0	R		
Cephalothin 160	-0 01	S	24	S	S	
Cephalothin 320	-0 01	S		S		
Tetracycline	+0.01	S	24	S	S	
Chloramphenicol	+0 01	S	32	S	S	
Clindamycin	+0.97	R	0	R	R	
Erythromycin	+0.66	R	13	R		
Gentamicin	-0.01	S	29	S	S	
Colistin	-0.01	S	17.5	S	S	
Kanamycin	-0.01	S	27	S	S	
Streptomycin	-0.01	S	22	S	S	
Nitrofurantoin	-0 01	S	23	S	S	
Usable Total = 13						
Sample No. 898, $A_0 = 5.84 \times 10^7$, $A_{2.5} = 1.43 \times 10^9$ >100,000 <i>Enterococcus</i>						
Penicillin	-0.02	S	17	I	I	(false S)
Ampicillin	-0.04	S	22	S	S	
Nafcillin	+1.05	R	0	R		
Cephalothin 160	+0.33	R	14	R	R	
Cephalothin 320	+0.01	S		R		
Tetracycline	+0.03	S	24	S	S	
Chloramphenicol	+0.04	S	26	S	S	
Clindamycin	+0 051	R	0	R	R	
Erythromycin	+0.03	S	26	S		
Gentamicin	+0.60	R	0*	R	S	
Colistin	+0 94	R	0	R	R	
Kanamycin	+0 67	R	0	R	R	
Streptomycin	+0.88	R	0	R	R	
Nitrofurantoin	+0.08	R	23	S	S	
Usable Total = 11						

* Disagreement

Table 5-5 (Continued)

Antibiotic	Index	ATP Interpretation	KB	K-B Interpretation	Laboratory Results	Disagreement
Sample No. 012, $A_0 = 4.82 \times 10^6$, $A_{2.5} = 2.20 \times 10^8$ >100,000 <i>E coli</i>						
Penicillin	+0.87	R	0	R	R	
Ampicillin	-0.02	S	18*	S	I	
Cephalothin 160	-0.01	S	19	S	S	
Cephalothin 320	-0.02	S	19	S		
Tetracycline	+0.04	S	20	S	S	
Chloramphenicol	+0.01	S	21, 32	S	S	
Clindamycin	+0.70	R	0	R	R	
Gentamicin	-0.02	S	23	S	S	
Colistin	-0.02	S	14	S	S	
Kanamycin	-0.02	S	20	S	S	
Streptomycin	+0.01	S	15*	S	R	
Nitrofurantoin	-0.01	S	20	S	S	
Usable Total = 11						
Sample No. 046, $A_0 = 5.76 \times 10^6$, $A_{2.5} = 2.20 \times 10^8$ >100,000 <i>E coli</i>						
Penicillin	+1.23	R	0	R	R	
Ampicillin	+1.25	R	8	R	R	
Cephalothin 160	+0.03	S	17*	I	S	
Cephalothin 320	-0.01	S	17*	I		
Tetracycline	+1.21	R	0	R	R	
Chloramphenicol	+0.01	S	25	S	S	
Clindamycin	+0.03	S	0	R	R	(false S)
Gentamicin	-0.02	S	22	S	S	
Colistin	-0.02	S	15	S	S	
Kanamycin	-0.02	S	19	S	S	
Streptomycin	+1.09	R	0	R	R	
Nitrofurantoin	-0.00	S	21	S	S	
Usable Total = 10						
Sample No. 977, $A_0 = 3.54 \times 10^6$, $A_{2.5} = 6.09 \times 10^7$ >100,000 <i>Enterococcus</i>						
Penicillin	-0.01	S	17	I	I	
Ampicillin	-0.05	S	22	S	S	
Cephalothin 160	+0.05	S	13	R	R	(false S)
Cephalothin 320	-0.03	S				
Tetracycline	+0.04	S	27	S	S	
Chloramphenicol	+0.04	S	22	S	S	
Clindamycin	+0.04	S	0	R	R	(false S)
Gentamicin	+0.73	R	13	S	S	(false R)
Colistin	+0.61	R	0	R	R	
Kanamycin	+0.81	R	0	R	R	
Streptomycin	+0.94	R	0	R	R	
Nitrofurantoin	+0.04	S	27	S	S	
Usable Total = 10						

* Disagreement

Table 5-5 (Continued)

Antibiotic	Index	ATP Interpretation	KB	K-B Interpretation	Laboratory Results	Disagreement	
Sample No. 524, $A_0 = 3.69 \times 10^6$, $A_{2.5} = 2.27 \times 10^8$ >100,000 <i>E. coli</i>							
Penicillin	+1.08	R	0	R	R	(false R)	
Ampicillin	-0.01	S	19	S	S		
Cephalothin 160	-0.01	S	21	S	S		
Cephalothin 320	-0.01	S					
Tetracycline	+0.01	S	23	S	S		
Chloramphenicol	+0.01	S	23	S	S		
Clindamycin	+0.89	R	0	R	R		
Gentamicin	-0.01	S	26	S	S		
Colistin	-0.02	S	16	S	S		
Kanamycin	-0.00	S	25	S	S		
Streptomycin	+0.07	R	22	S	S		
Nitrofurantoin	-0.01	S	23	S	S		
					Usable Total = 11		
Sample No. 588, $A_0 = 3.76 \times 10^6$, $A_{2.5} = 1.80 \times 10^8$ >100,000 <i>Klebsiella</i>							
Penicillin	+0.99	R	0	R	R		
Ampicillin	+0.99	R	0	R	R		
Cephalothin 160	-0.02	S	23	S	S		
Cephalothin 320	-0.02	S					
Tetracycline	+0.01	S	23	S	S		
Chloramphenicol	+0.02	S	29	S	S		
Clindamycin	+0.98	R	0	R	R		
Gentamicin	-0.02	S	24	S	S		
Colistin	-0.02	S	13	S	S		
Kanamycin	-0.02	S	22	S	S		
Streptomycin	+0.00	S	19	S	I		
Nitrofurantoin	-0.01	S	21	S			
					Usable Total = 11		
Sample No. 662, $A_0 = 3.64 \times 10^6$, $A_{2.5} = 1.19 \times 10^8$ >100,000 <i>E. coli</i>							
Penicillin	+1.20	R	0	R	R		
Ampicillin	-0.02	S	19	S	S		
Cephalothin 160	-0.01	S	21	S	S		
Cephalothin 320	-0.03	S					
Tetracycline	+0.04	S	22	S	S		
Chloramphenicol	+0.05	S	28	S	S		
Clindamycin	+1.61	R	0	R	R		
Gentamicin	-0.03	S	25	S	S		
Colistin	-0.03	S	15	S	S		
Kanamycin	-0.02	S	23	S	S		
Streptomycin	-0.01	S	20	S	S		
Nitrofurantoin	-0.00	S	22	S			
					Usable Total = 11		

Table 5-5 (Continued)

Antibiotic	Index	ATP Interpretation	KB	K-B Interpretation	Laboratory Results	Disagreement
Sample No 861, $A_0 = 2.64 \times 10^6$, $A_{2.5} = 1.74 \times 10^8$ >100,000 <i>Enterobacter</i> #1, >100,000 <i>Enterobacter</i> #2						
			No. 1	No. 2	No. 1	No. 2
Penicillin	+1.02	R	0	0	R	R
Ampicillin	+0.36	R	13	13	I	I
Cephalothin 160	+0.11	R	13	13.5	R	R
Cephalothin 320	+0.03	S				
Tetracycline	+0.01	S	23	19	S	S
Chloramphenicol	+0.01	S	29	28	S	S
Clindamycin	+0.85	R	0	0	R	R
Gentamicin	-0.01	S	25	25	S	S
Colistin	-0.01	S	15	15	S	S
Kanamycin	-0.01	S	23	22	S	S
Streptomycin	+0.00	S	14, 20	22	I	S
Nitrofurantoin	-0.00	S	19	18	S	S
Usable Total = 10						(false S)
Sample No. 878, $A_0 = 5.83 \times 10^6$, $A_{2.5} = 2.99 \times 10^8$ >100,000 <i>Enterococcus</i>						
Penicillin	+0.92	R	0	R	R	
Ampicillin	+0.98	R	0	R	R	
Cephalothin 160	+0.92	R	0	R	R	
Cephalothin 320	+0.94	R				
Tetracycline	+0.01	S	23	S	S	
Chloramphenicol	-0.01	S	29	S	S	
Clindamycin	+0.99	R	0	R	R	
Gentamicin	-0.02	S	26	S	S	
Colistin	-0.02	S	15	S	S	
Kanamycin	-0.02	S	23	S	S	
Streptomycin	-0.00	S	20	S	S	
Nitrofurantoin	-0.01	S	21	S	S	
Usable Total = 11						
Sample No 927; $A_0 = 1.30 \times 10^6$, $A_{2.5} = 1.21 \times 10^7$ 30 - 50,000 <i>Staphylococcus</i> with diphtheroids						
Penicillin	-0.04	S	14	R	R	(false S)
Ampicillin	-0.07	S	15	R	R	(false S)
Cephalothin 160	-0.04	S	30	S	S	
Cephalothin 320	-0.01					
Tetracycline	+0.10	R	28	S	S	(false R)
Chloramphenicol	+0.10	R	26	S	S	(false R)
Clindamycin	-0.01	S	20	S	R	
Gentamicin	+0.01	S	23	S	S	
Colistin	+0.76	R	0	R	R	
Kanamycin	+0.02	S	20	S	S	
Streptomycin	+0.31	R	15	S	I	(false R)
Nitrofurantoin	-0.00	S	19	S	S	
Usable Total = 11						

Table 5-6
Zone Diameter Criteria for Assigning Resistance or Sensitivity

		<u>Sensitive</u> (disc size mm $\geq$)	<u>Indeterminate</u>	<u>Resistant</u> (disc size mm $\leq$)
Penicillin	for <i>Staphylococcus</i>	29		20
	for gram negatives	22		11
Ampicillin	for <i>Staphylococcus</i>	29		20
	for gram negatives	14		11
Nafcillin		14		9
Cephalothin		18		14
Tetracycline		19		14
Chloramphenicol		18		12
Lincomycin (Clindamycin)		15		10
Erythromycin		18		13
Gentamicin		13		12
Colistin		11		8
Kanamycin		18		13
Streptomycin		15		11
Nitrofurantoin		17		14

There are certainly a number of factors that could give rise to disagreements between the ATP method and Kirby-Bauer method, and some are listed as follows:

- Problems secondary to antibiotic mechanisms and expressed by prevalence of certain organism-antibiotic combinations
- Inoculum size
- Limited growth of organisms over 2.5 hours
- Presence of white blood cells in the urine
- Clinical use of antibiotics

Antibiotic Mechanism

Table 5-7 lists errors that occurred for each antibiotic tested. The sample number, organism name, and number of organisms per ml by plate count are listed. Errors that occurred for each sample are given across the page from left to right. False-resistant (R) or false-sensitive (S) as defined previously are indicated along with the ATP index.

It is immediately apparent that a majority of errors occurred with ampicillin and cephalothin. Considering only nonmixed samples, of 27 total errors, 6 occurred with ampicillin. All errors were false-resistant. Two errors occurred with *Proteus*, three errors with *E. coli*, and one error with *Klebsiella*. Indices were in the low or middle range except for sample 524, which had an index of 0.94.

The problem of false resistance with ampicillin-*Proteus* had been identified in previous work and was attributed to antibiotic mechanisms. The reason for the problem with ampicillin-*E. coli* combination is not clear.

Considering nonmixed specimens only, 6 of 27 total errors occurred with cephalothin. Three cases involved *E. coli*, two cases, *Proteus*, and one case, *Enterococcus*. All cases except sample 977, *Enterococcus*, were false-resistant. The indices were in a low or middle range in all cases of false-resistance. Some problems with the cephalothin-*Proteus* combination had been observed in previous work, but an explanation for the cephalothin-*E. coli* combination problem is not clear.

Nitrofurantoin contributed 4 of 27 errors. All except one were false-resistant with indices in the low or middle range. Three of four errors occurred with the nitrofurantoin-*Klebsiella* combination.

The remaining 10 errors were spread throughout the remaining antibiotics. The majority of errors were again false-resistant.

Correlation for individual antibiotics, considering only nonmixed specimens, was as follows

<u>Antibiotic</u>	<u>Percent Correlation</u>
Penicillin	99.9
Ampicillin	82.4
Cephalothin	82.4
Tetracycline	97.0
Chloramphenicol	94.0
Clindamycin	94.3
Erythromycin	100.0

<u>Antibiotic</u>	<u>Percent Correlation</u>
Gentamicin	96.9
Colistin	100.0
Kanamycin	100.0
Streptomycin	88.9
Nitrofurantoin	88.2

Inoculum Size

Previous work had established that antibiotic susceptibility testing could be carried out successfully with inoculum sizes of 1×10^5 or 1×10^6 organisms/ml. Inoculum sizes outside this range might be a source of error. With these samples, the ATP level obtained from a detection ATP assay (by the long centrifugation method) was used to dilute the bacterial sediment in an attempt to achieve appropriate inoculum sizes. In all but three cases, the A_0 light units fell between 1×10^5 and 1×10^7 . In order to determine if inoculum size altered correlation between the ATP method and the Kirby-Bauer, nonmixed samples were grouped into four ranges according to light units: 1.0×10^5 to 1.0×10^6 , 1.0×10^6 to 5.0×10^6 ; 5.0×10^6 to 1.0×10^7 ; and $> 1.0 \times 10^7$. Table 5-8 gives the results. For the three lower ranges, correlations were not significantly different, 93.2, 92.9 and 95.8 percent. For the three samples with A_0 's $> 1.0 \times 10^7$, the correlation was lower at 85.7 percent. Because of the limited number of samples involved, this does not prove that high A_0 's are a source of error. Further clinical trials must attempt to clarify this problem.

Limited Growth of Organisms over 2.5 Hours

It had been observed earlier that when only a small amount of growth occurred in the growth control, results did not correlate well with Kirby-Bauer. To test the influence of this factor on results, samples were grouped as to the amount of growth that occurred in the growth control. Table 5-9 gives these groupings. Fold increase in the fourth column was calculated by dividing $A_{2.5}$ by A_0 . Samples were grouped into three ranges as indicated: 0 to 30; 30 to 60; and 60-fold increase.

The group of samples in the 0 to 30-fold increase range did not have as high a correlation as the higher ranges, 89.3 percent compared to 95.2 and 93.2 percent.

Presence of White Blood Cells in the Urine

The possibility that white blood cells in the urine might interfere with susceptibility tests was of great concern. To examine this possibility, samples were grouped according to white blood cell counts in the urine.

Table 5-7
False-Resistant (R) and False-Sensitive (S) Indices Obtained
in the Final Clinical Trial of ATP Method

Sample Number	Organism Name and Number per ml by Plate Count	Antibiotic													
		Pen	Amp	Naf	Carb	Ceph	Tetra	Chlora	Clinda	Erythro	Genta	Colliath	Kana	Strept	Nitro
967	>100,000 <i>P mirabilis</i> <10,000 coliform		(R) + 12												
946	>100,000 <i>Klebsiella</i>		(R) + 18												
363	>100,000 <i>P rettgeri</i>		(R) + 29			(R) + 56									
361	>100,000 <i>E. coli</i>		(R) + 07			(R) + 10									
650	>100,000 <i>E. coli</i>														
739	30-50,000 <i>E. coli</i> < 10,000 <i>Proteus</i> and <i>Enterococcus</i>					(S) + 01	(S) + 02								(S) - 01
749	>100,000 <i>P rettgeri</i>		(R) + 16			(R) + 42									
191	>100,000 <i>E. coli</i>		(R) + 26			(R) + 20								(R) + 16	(R) + 13
294	>100,000 <i>Klebsiella</i>														
524	>100,000 <i>E. coli</i>		(R) + 94	(S) - 05		(R) + 10	(R) + 07								(R) + 13
751	>100,000 <i>Klebsiella</i>														
774	50-100,000 <i>P mirabilis</i>														
466	>100,000 <i>E. coli</i>														
512	>100,000 <i>E. coli</i>					(R) + 14				(R) + 08					
860	>100,000 <i>Klebsiella</i>														
898	>100,000 <i>Enterococcus</i>														(R) + 34
046	>100,000 <i>E. coli</i>														(R) + 08
977	>100,000 <i>Enterococcus</i>					(S) + 05		(S) + 03							
524 ¹	>100,000 <i>E. coli</i>						(R) + 10	(S) + 04		(R) + 73				(R) + 07	
927	30-50,000 <i>Staphylococcus diptheroidea</i>	(S) - 04	(S) - 07				(R) + 10	(R) + 10						(R) + 31	

Table 5-8
Analysis of Errors by A_0 Values (Nonmixed infections)

Specimen Number	Organism (Name and Number/ml)	A_0 Light Units	$A_{2.5}$ Light Units	Fold Increase	Usable Total	Errors	% Agreement with K-B
$1.0 \times 10^5 - 1.0 \times 10^6$							
101	>100,000 <i>Pseudomonas</i>	5.30×10^5	5.77×10^6	10.9	10	0	
200	>100,000 <i>E coli</i>	4.50×10^5	5.04×10^7	112.0	11	0	
363	>100,000 <i>P reitgeri</i>	5.07×10^5	1.37×10^7	27.0	11	2	
361	>100,000 <i>E coli</i>	3.63×10^5	2.57×10^7	70.8	9	2	
584	>100,000 <i>Proteus</i>	6.53×10^5	2.65×10^6	4.1	12	1	
994	>100,000 <i>E coli</i>	9.14×10^5	3.61×10^7	39.5	13	0	
997	>100,000 <i>E coli</i>	3.99×10^5	2.12×10^7	53.1	13	0	
751	>100,000 <i>Klebsiella</i>	9.88×10^5	4.33×10^7	43.8	12	1	
774	50-100,000 <i>P mirabilis</i>	8.57×10^5	3.43×10^7	40.0	12	1	
					103	7	93.3%
$1.0 \times 10^6 - 5.0 \times 10^6$							
662	>100,000 <i>E coli</i>	3.64×10^6	1.19×10^8	32.7	11	0	
946	>100,000 <i>Klebsiella</i>	1.90×10^6	2.30×10^8	121.1	11	1	
229	50-100,000 <i>Proteus</i>	1.60×10^6	3.01×10^7	18.8	9	0	
650	>100,000 <i>E coli</i>	3.26×10^6	1.42×10^8	43.6	11	1	
749	>100,000 <i>P reitgeri</i>	1.92×10^6	7.15×10^7	37.2	13	2	
191	>100,000 <i>E coli</i>	4.63×10^6	6.69×10^7	14.4	12	1	
504	>100,000 <i>E coli</i>	3.69×10^6	2.82×10^8	76.4	12	0	
512	>100,000 <i>E coli</i>	3.88×10^6	1.33×10^8	34.3	12	1	
860	>100,000 <i>Klebsiella</i>	4.49×10^6	3.35×10^8	74.6	11	1	
883	>100,000 <i>E coli</i>	4.23×10^6	2.69×10^8	63.6	11	0	
012	>100,000 <i>E coli</i>	4.82×10^6	2.20×10^8	45.6	11	0	
997	>100,000 <i>Enterococcus</i>	3.54×10^6	6.09×10^7	17.2	10	3	
524	>100,000 <i>E coli</i>	3.69×10^6	2.29×10^8	61.5	11	1	
588	>100,000 <i>Klebsiella</i>	3.76×10^6	1.80×10^8	47.9	11	0	
					156	11	92.9%

Table 5-8 (Continued)

Specimen Number	Organism (Name and Number/ml)	A ₀ Light Units	A _{2.5} Light Units	Fold Increase	Usable Total	Errors	% Agreement with K-B
$5.0 \times 10^6 - 1.0 \times 10^7$							
232	>100,000 <i>Klebsiella</i>	8.58×10^6	3.34×10^8	38.9	12	0	
256	>100,000 <i>Serratia marcescens</i>	6.63×10^6	2.44×10^8	36.8	12	0	
294	>100,000 <i>Klebsiella</i>	5.94×10^6	4.36×10^8	73.4	12	1	
466	>100,000 <i>E. coli</i>	5.73×10^6	2.16×10^8	37.7	12	1	
795	>100,000 <i>E. coli</i>	9.48×10^6	3.77×10^8	39.8	13	0	
837	>100,000 <i>Staph. megati</i>	5.76×10^6	5.58×10^7	9.7	13	0	
046	>100,000 <i>E. coli</i>	5.76×10^6	2.20×10^8	38.2	10	2	
588	>100,000 <i>Enterococcus</i>	5.83×10^6	2.99×10^8	51.3	11	0	
					95	4	95.8%
1.0×10^7							
524	>100,000 <i>E. coli</i>	1.36×10^7	2.19×10^8	16.1	12	4	
817	>100,000 <i>E. coli</i>	2.30×10^7	5.00×10^8	21.7	12	0	
898	>100,000 <i>Enterococcus</i>	5.84×10^7	1.43×10^9	24.5	11	1	
					35	5	85.7%

Table 5-9
Analysis of Errors by Fold Increase (Nonmixed infection)

Specimen Number	Organism (Name and Number/ml)	Fold Increase	Usable Total	Errors	% Agreement with K-B
<u>0 to 30.0 fold increase</u>					
584	>100,000 <i>Proteus</i>	4.1	12	1	
837	>100,000 <i>Staphylococcus megati</i>	9.7	13	0	
101	>100,000 <i>Pseudomonas</i>	10.9	10	0	
191	>100,000 <i>E coli</i>	14.4	12	1	
524	>100,000 <i>E coli</i>	16.1	12	4	
997	>100,000 <i>Enterococcus</i>	17.2	10	3	
229	50-100,000 <i>Proteus</i>	18.8	9	0	
817	>100,000 <i>E coli</i>	21.7	12	0	
898	>100,000 <i>Enterococcus</i>	24.5	11	1	
363	>100,000 <i>P rettgeri</i>	27.0	11	2	
			<u>112</u>	<u>12</u>	89.3%
<u>30.0 to 60.0 fold increase</u>					
662	>100,000 <i>E coli</i>	32.7	11	0	
512	>100,000 <i>E coli</i>	34.3	12	1	
256	>100,000 <i>Serratia marcesens</i>	36.8	12	0	
749	>100,000 <i>P rettgeri</i>	37.1	13	2	
466	>100,000 <i>E coli</i>	37.7	12	1	
046	>100,000 <i>E coli</i>	38.2	10	2	
232	>100,000 <i>Klebsiella</i>	38.9	12	0	
994	>100,000 <i>E coli</i>	39.5	13	0	
795	>100,000 <i>E coli</i>	39.8	13	0	
774	50-100,000 <i>P mirabilis</i>	40.0	12	1	
650	>100,000 <i>E coli</i>	43.6	11	1	
751	>100,000 <i>Klebsiella</i>	43.8	12	1	
012	>100,000 <i>E coli</i>	45.6	11	0	
588	>100,000 <i>Klebsiella</i>	47.9	11	0	
898	>100,000 <i>Enterococcus</i>	51.3	11	0	
997	>100,000 <i>E coli</i>	53.1	13	0	
			<u>189</u>	<u>9</u>	95.2%

Table 5-9 (Continued)

Specimen Number	Organism (Name and Number/ml)	Fold Increase	Usable Total	Errors	% Agreement with K-B
<u>≥60.0 fold increase</u>					
524	>100,000 <i>E coli</i>	61.5	11	1	
883	>100,000 <i>E coli</i>	63.6	11	0	
361	>100,000 <i>E coli</i>	70.8	9	2	
294	>100,000 <i>Klebsiella</i>	73.4	12	1	
860	>100,000 <i>Klebsiella</i>	74.6	11	1	
504	>100,000 <i>E coli</i>	76.4	12	0	
200	>100,000 <i>E coli</i>	112.0	11	0	
946	>100,000 <i>Klebsiella</i>	121.1	11	1	
			<u>88</u>	<u>6</u>	93.2%

Of interest is the distribution of white blood cell counts. Of 35 urines with nonmixed infections, only 5 had white blood cell (wbc) counts greater than 1×10^6 /ml. The highest count encountered was 3.0×10^6 wbc/ml. Thirteen of 35 urines had counts in the range of $1.0 \times 10^5 - 1.0 \times 10^6$ wbc/ml. Seventeen of the 35 urines had less than 1.0×10^5 wbc/ml in the urine.

Table 5-10 gives the specimen number, wbc/ml A_0 light units for the sample, total antibiotic determinations, and number of errors. Samples that had less than 1.0×10^5 wbc/ml had a 91.9-percent correlation with the Kirby-Bauer method. Samples with counts between 1.0×10^5 and 1.0×10^6 wbc/ml had a 94.7-percent correlation. Samples with counts greater than 1.0×10^6 wbc/ml had a 95.2-percent correlation. Certainly no trend towards decreasing correlation with increasing white blood cell counts was found; in fact, the best results were obtained with urines with the higher white blood cell counts.

To review briefly, the ATP susceptibility test relies on an increase in ATP over time. Therefore, even if nonbacterial ATP falsely elevates the A_0 determination, it will elevate the A_{25} by the same increment. This constant contribution will usually become mathematically insignificant when the ratio calculation is made. Secondly, significant pyurias are usually accompanied by high bacteria counts. The sample will usually be diluted appropriately on the basis of the ATP level obtained from the detection assay (long centrifugation) which can handle at least 1.0×10^6 wbc/ml. When dilution is carried out, the number of white blood cells is proportionately reduced, and the contribution to the A_0 decreases. This was confirmed in the clinical samples because high white blood cell counts did not result in higher than normal A_0 's.

Clinical Use of Antibiotics

In our preliminary work, using patient urine samples inoculated with various reference strains of bacteria, clinical use of antibiotics, had interfered with susceptibility testing. This was thought to be an artificial problem. If a patient developed a urinary tract infection while on antibiotics, the organism would be resistant to the antibiotic in the urine. Growth would occur in the growth control and susceptibility testing could be carried out.

We encountered four patients on antibiotics with significant bacteriurias. Sample numbers 978 and 994 (table 5-4) were from patients on ampicillin. Sample number 256 was from a patient on gantrisin, and sample number 294 was from a patient on cephalothin. Only one error occurred for 50 determinations from these samples giving a high correlation of 98.0 percent. Our impression that susceptibility testing can be carried out when the patient is already on antibiotics was preliminarily confirmed.

In the study of clinical urine specimens, samples were selected that appeared by plate count to contain only one organism. In seven samples a second organism was eventually recognized. The samples were numbers 967, 040, 640, 739, 978, 168, and 927. In all except three samples, the dominant organism was present at a level of greater than 100,000 organisms/ml, and the second organism was present at a level of less than 10,000 organisms/ml. Sample number 978 contained *E coli* at 50,000 to 100,000 organisms/ml and diptheroids at $< 10,000$

Table 5-10

Errors According to White Blood Cell Counts in the Urine (Nonmixed)

Specimen Number	wbc/ml	Light ^{A0} Units	Usable Total	Errors	% Agreement with K-B	
< 1.0 × 10 ⁵						
040	occasional	3.49 × 10 ⁵	10	0	91.9%	
200	rare	4.50 × 10 ⁵	11	0		
229	rare	1.60 × 10 ⁵	9	0		
361	5.0 × 10 ⁴	3.63 × 10 ⁵	9	2		
584	3.0 × 10 ⁴	6.53 × 10 ⁵	12	0		
466	<10 ⁴	5.73 × 10 ⁶	12	1		
504	0	3.69 × 10 ⁶	13	0		
524	7.0 × 10 ⁴	1.36 × 10 ⁷	12	4		
837	occasional	5.76 × 10 ⁶	13	0		
860	7.0 × 10 ⁴	4.49 × 10 ⁶	11	1		
898	5.0 × 10 ⁴	5.84 × 10 ⁷	11	1		
046	occasional	5.76 × 10 ⁶	10	0		
977	1.0 × 10 ⁴	3.54 × 10 ⁶	10	3		
524	occasional	3.69 × 10 ⁶	11	1		
588	0	3.76 × 10 ⁶	11	0		
861	4.0 × 10 ⁴	2.64 × 10 ⁶	10	1		
878	occasional	5.83 × 10 ⁶	11	0		
			186	15		
1.0 × 10 ⁵ - 1.0 × 10 ⁶						
246	2.4 × 10 ⁵	1.90 × 10 ⁶	11	1		
101	1.0 × 10 ⁵	5.30 × 10 ⁵	10	0		
363	1.0 × 10 ⁵	5.07 × 10 ⁵	11	2		
650	5.1 × 10 ⁵	3.26 × 10 ⁶	11	1.		
997	2.2 × 10 ⁵	3.99 × 10 ⁵	13	0		
191	1.0 × 10 ⁵	4.63 × 10 ⁶	12	1		
232	1.5 × 10 ⁵	8.58 × 10 ⁶	12	0		
294	1.0 × 10 ⁵	5.94 × 10 ⁶	12	1		
751	1.2 × 10 ⁵	9.87 × 10 ⁵	12	1		

Table 5-10 (Continued)

Specimen Number	wbc/ml	A_0 Light Units	Usable Total	Errors	% Agreement with K-B
774	2.3×10^5	8.57×10^5	12	1	94.7%
817	6.6×10^5	2.30×10^7	12	0	
883	3.4×10^5	4.23×10^6	13	0	
662	7.2×10^5	3.64×10^6	11	0	
			152	8	
$>1.0 \times 10^6$					
749	3.0×10^6	1.93×10^6	13	2	95.2%
994	1.2×10^6	9.14×10^5	13	0	
512	1.5×10^6	3.88×10^6	12	1	
795	1.0×10^6	9.48×10^6	13	0	
012	2.0×10^6	4.82×10^6	11	0	
			62	3	

organisms/ml. Sample number 739 contained 30,000 to 50,000 *E. coli* and < 10,000 *Proteus*. Sample number 168 contained *E. coli* at > 100,000 organisms/ml and 30,000 to 50,000 *P. mirabilis*.

Agreement between the ATP method and the Kirby-Bauer method for these seven mixed samples was 89.8 percent, which was only slightly lower than the overall agreement of 92.8 percent. It appears that susceptibility tests can be carried out with good correlation with mixed infections when one organism greatly dominates the other.

SUMMARY

In summary, the clinical studies incorporating the changes outlined in this section established that an ATP assay suitable for detection of bacteriurias could be combined successfully with susceptibility testing, that results from an ATP assay suitable for detection of bacteriuria could be used to establish appropriate bacterial inoculum sizes for susceptibility testing, and that certain technical modifications representing considerable time and equipment savings could be used without obvious decrease in correlation of results.

Our analysis of errors identified the following factors worthy of additional consideration:

- Particular antibiotics were responsible for the majority of errors (ampicillin and cephalothin). The ampicillin-*Proteus* combination was again recognized as a source of false-resistant results. Future work should evaluate these problem antibiotics, and possible solutions should be incorporated into further clinical trials. One hopeful possibility would be to lengthen the incubation period to 3 hours to solve the ampicillin problem.
- High A_0 's may be a source of error and this factor should be examined with further clinical trials.
- Mixed cultures gave workable results in this study when one organism dominated. Future work must evaluate mixed cultures in which more equal numbers of two organisms are found. (Shahidi, Ellner, 1969)
- White blood cells in the urines did not interfere with the ATP antibiotics susceptibility testing at levels encountered in this clinical trial. Urines with higher white blood cell counts should be sought and evaluated.

Concerning overall success of the method, the 92.5-percent correlation for all samples is in agreement with that obtained by Vellend in the pure species work. It is confirmed that the ATP antibiotic susceptibility testing methods can be applied directly to urine specimens.

ACKNOWLEDGMENTS

We wish to acknowledge Dr. David Rutstein of the Harvard Medical School for the considerable perception and judgment he brought to bear in the original conception of rapid antibiotic susceptibility test that would use the firefly luciferase ATP assay. His constant interest in the progress of the developmental research has been greatly appreciated. Financial backing for the project was obtained in large part by the efforts of Robert Zimmerman of NASA Headquarters, whose overt interest and recognition of the value of the research assured continued support and funding. E. I. DuPont de Nemours and Co., Inc., was kind enough to loan Luminescence 760 Biometers and to supply us with substantial amounts of purified firefly luciferase.

LIST OF PATENTS

ISSUED

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APPENDIX A

SUMMARY OF STUDIES APPLYING RAPID MICROBIAL SENSITIVITY TEST USING ATP TO PURE BACTERIAL CULTURES

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APPENDIX A

SUMMARY OF STUDIES APPLYING RAPID MICROBIAL SENSITIVITY TEST USING ATP TO PURE BACTERIAL CULTURES

INTRODUCTION

Optimum use of antimicrobial agents to treat infection depends upon knowledge of the susceptibility of the infecting organism. Although susceptibility can sometimes be predicted from the general behavior of the bacterial species involved, the most reliable approach is generally that of direct measurement of the *in vitro* interaction between the organism and the drug.

Present techniques of microbial sensitivity testing generally require overnight incubation after primary isolation of the infecting organism. This requires a minimum of 48 hours after the specimen is received in the laboratory. The methods most widely used are agar diffusion (Kirby-Bauer), broth dilution, and agar dilution. Only the agar diffusion technique is reasonably well standardized and is widely used in clinical microbiology laboratories. This delay in providing the physician with accurate susceptibility data often results in the institution of empirical and/or broad-spectrum antimicrobial therapy in urgent clinical situations. The *in vivo* patient response to this therapy can often be measured before the laboratory is able to provide *in vitro* sensitivity data. The availability of a rapid method of testing bacterial susceptibility would permit prompt selection of the most effective agent and therefore avoid the inclusion of inappropriate, unnecessary, and often toxic agents in the initial therapeutic regimen.

Several rapid methods of microbial susceptibility testing, incorporating a wide variety of techniques of measuring antimicrobial effect, have been described in the past few years. We have chosen to measure ATP as an indicator of antibiotic effect for several reasons: (1) this substance is present in all viable microorganisms; (2) its concentration in bacteria is directly related to fundamental processes of oxidative phosphorylation and respiration; (3) although none of the commonly used antimicrobial agents acts directly on oxidative phosphorylation and respiration, it is evident that they may have profound indirect consequences on the ATP content of bacteria; and (4) the measurement of ATP by the luciferase bioluminescence reaction is precise, reproducible, and compatible with automation. The assay can be made specific for bacterial ATP by chemically removing the ATP associated with nonbacterial cells as well as any soluble ATP. This is accomplished by treating the specimen with a nonionic detergent, Triton X-100. Under the conditions used in our experiments, this detergent selectively lyses mammalian cells without effecting any change in the ATP content of bacteria. An ATPase is then added which hydrolyzes all the free ATP. An individual specimen can be assayed for its ATP content by this method in less than 30 minutes.

In conventional methods of testing susceptibility to antimicrobial agents, the end-point is measured after 18 to 24 hours of interaction between drug and microorganism. Any method which attempts to assess this interaction at a shorter interval offers the distinct possibility

that results may differ from those obtained by the longer method. The majority of the discrepancies will arise from three factors: (1) the particular characteristic of the bacterium being measured; in this case, bacterial ATP; (2) the mode of action of the antimicrobial agent; and (3) the mechanism of resistance to the antimicrobial agent.

MATERIALS AND METHODS

Bacterial Strains

The majority of the bacterial strains used in this study were clinical isolates obtained from the diagnostic microbiology laboratory at the New England Medical Center Hospital. Reference strains were obtained from the American Type Culture Collection (*Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923) and from Difco Laboratories Bac-trol Discs (*Klebsiella aerogenes* ATCC 23357, *Enterobacter cloacae* ATCC 23355, *Serratia marcescens* ATCC 8100, *Proteus vulgaris* ATCC 6380, *Pseudomonas aeruginosa* ATCC 14207). Each strain was characterized by standard laboratory techniques. The following number of strains of each species were studied: *Escherichia coli* (11), *Klebsiella aerogenes* (10), *Enterobacter cloacae* (6), *Serratia marcescens* (6), *Proteus mirabilis* (11), *Proteus vulgaris* (2), *Providencia stuartii* (1), *Pseudomonas aeruginosa* (11), *Staphylococcus aureus* (18), *Staphylococcus epidermidis* (2), and *Enterococcus* (11).

These organisms were maintained on trypticase soy agar slants at 4°C. At monthly intervals, a fresh broth culture was streaked out on 5-percent sheep's blood agar and eosinmethylene blue agar to confirm purity. Five similar colonies from the blood agar were inoculated into fresh broth from which new agar slants were prepared. Agar diffusion sensitivity tests were done periodically on these strains to confirm stability of their susceptibility patterns.

Antibiotics

Standard antibiotic powders suitable for sensitivity testing were obtained from manufacturers: Bristol Laboratories, Syracuse, New York—ampicillin trihydrate and tetracycline hydrochloride; Eli Lilly and Company, Indianapolis, Indiana—potassium penicillin G, sodium cephalothin and erythromycin base; Warner-Chilcott Laboratories, Morris Plains, New Jersey—sodium colistimethate; Eaton Laboratories, Norwich, New York—nitrofurantoin; Wyeth Laboratories, Philadelphia, Pennsylvania—sodium nafcillin and sodium ampicillin, Winthrop Laboratories, New York, New York—nalidixic acid; Parke, Davis and Company, Detroit, Michigan—chloramphenicol; Roerig, New York, New York—disodium carbenicillin; Schering Corporation, Bloomfield, New Jersey—gentamicin sulfate; Upjohn Company, Kalamazoo, Michigan—clindamycin phosphate; Hoffmann-LaRoche Inc., Nutley, New Jersey—sulfisoxazole. All standard powders were stored in a vacuum desiccator at 4°C.

To prepare stock solutions (1280 µg/ml), these powders were dissolved in sterile distilled water and dispensed into sterile, capped polypropylene tubes (12 × 75 mm) using a Cornwall syringe pipette with a 0.22-µm Millipore filter. Special solvents were used in small amounts for sulfisoxazole (10-percent sodium hydroxide), erythromycin base (ethanol), nitrofurantoin

(dimethylformamide, as per manufacturers' instructions), and nalidixic acid (1N sodium hydroxide). These solutions were frozen at -20°C for no longer than 2 weeks and used within 1 hour of being thawed.

The antibiotics studied and the concentration used to determine ATP index are shown in table A-1. The concentration selected was determined empirically by measuring the effect of the mean inhibitory concentration (MIC) breakpoints generally quoted for the agar diffusion method on a panel of prototype organisms. The concentrations shown in table A-1 fall somewhere in between these MIC breakpoints.

Table A-1
Comparison of Results Obtained by the ATP
Index and Agar Diffusion Sensitivity Testing

Antibiotic	Concentration µg/ml	Total Strains	DISAGREEMENT					
			Total	(%)	Major	False		Minor
						Resistant	Sensitive	
Ampicillin	8	69	13	(19)	13	12	1	0
Penicillin G	8	69	10	(14)	10	10	0	0
	0.075*	18	1	(5)	1	1	0	0
Nafcillin	0.6	2	0	(0)	0	0	0	0
Carbenicillin	128	33	7	(21)	4	4	0	3
Cephalothin	16	82	4	(5)	4	3	1	0
Tetracycline	6	81	7	(9)	2	1	1	5
Erythromycin	4	77	4	(5)	0	0	0	4
Clindamycin	2	78	9	(12)	9	0	9	0
Gentamicin	6	81	0	(0)	0	0	0	0
Nitrofurantoin	50	83	19	(23)	13	12	1	6
Colistin	8	81	2	(2)	2	0	2	0
Chloramphenicol	12.5	81	7	(9)	5	3	2	2
	TOTAL	835	83	(10)	63	46	17	20

**S. aureus* strains only.

Agar Diffusion Sensitivity Testing

Agar diffusion sensitivity testing was performed according to the tentative standards recommended by the National Committee for Clinical Laboratory Standards Subcommittee on Antimicrobial Susceptibility Testing.

Broth Dilution MIC's

This procedure was done in trypticase soy broth according to the method proposed by the International Collaborative Study on Antibiotic Sensitivity Testing.

ATP Index

Method of Incubation

A fresh overnight broth culture is diluted 1000-fold in trypticase soy broth at 37°C to obtain an inoculum of approximately 10^6 colony-forming units (cfu)/ml. This is preincubated for 30 minutes at 37°C and 4.5 ml was added to each of two tubes, tube A containing 0.5 ml of sterile 0.9-percent sodium chloride (growth control), and tube B containing 0.5 ml of an antibiotic solution in 0.9-percent sodium chloride to yield the final concentration shown in table A-1. From control tube, A, 0.5 ml of the broth culture is immediately assayed for ATP (A_0), and both tubes are incubated at 37°C for 2.5 hours; 0.5-ml samples from each tube are then assayed for ATP (A_t and B_t).

Method of ATP Assay for Pure Bacterial Species in Broth

Because small amounts of ATP are ubiquitous in untreated water, it is necessary to use distilled, deionized water in preparing all reagents. ATP clings to glassware, therefore all glassware must be acid cleaned. Plastic ware is generally preferable. Many commercial detergents will interfere with the assay, and glassware must be scrupulously free of residue.

Two basic reagents are used:

- **Apy-Ca-Tx**—A solution of 0.03 M calcium chloride and 0.6-percent Triton X-100 is kept frozen at -20°C in aliquots suitable for a day's work. Five mg ATPase (Apyrase, Grade 1, Sigma) per ml are added to this solution immediately prior to performance of the assay and gently mixed by inversion.
- **Luciferase**—The contents of one vial of luciferase (Luminescence Biometer Reagent Kit, DuPont Instruments) are reconstituted with 1.5 ml of 0.2 M TRIS (Trizma base, Sigma) containing 0.01 M magnesium sulfate (pH 8.4), which is also kept frozen in suitable aliquots. After complete solution, the reagent is dispensed (0.1 ml) into reaction cuvettes (6 × 50 mm).

The assay is performed in the following steps:

1. Place a 0.5-ml sample of the broth culture in a sterile polypropylene tube (17 × 100 mm).
2. Add 0.1 ml of Apy-Ca-Tx. Vortex well and wait 15 minutes.
3. Add exactly 0.1 ml of 1.5 N nitric acid. Vortex tube and allow to stand for 5 minutes.
4. Add 4.3 ml of sterile, deionized water (Travenol, sterile water for irrigation) and mix well.
5. Draw 0.1 ml of the reaction mixture into a disposable tuberculin syringe and inject into the luciferase reagent.
6. Readings are made in a photometer (DuPont Luminescence 760 Biometer) standardized to 1.00×10^8 femtograms (fg) ATP/ml of original sample by adding 0.05 ml of a freshly thawed ATP standard (1.0 µg/ml) to the final reaction volume of a blank tube (sterile broth).

Calculation of ATP Index

The drug effect on bacterial ATP content was quantitated using the following formula:

$$\text{ATP Index} = \frac{\log B_t - \log A_0}{\log A_t - \log A_0}$$

the current interpretation of the ATP index is as follows:

>+ 0.25 Resistant

≤+ 0.25 Sensitive

Reproducibility Studies

A limited study was undertaken to determine the reproducibility of the ATP index. The same 10 bacterial cultures (*E. coli* ATCC 25922, *Klebsiella Pneumoniae* ATCC 23357, *Enterobacter cloacae* ATCC 23355, *Serratia marcescens* ATCC 8100, *Proteus vulgaris* ATCC 6380 and NEMCH 528, *Pseudomonas aeruginosa* ATCC 14207, *Providencia stuartii* NEMCH 321, *Staphylococcus aureus* ATCC 25923, and *Staphylococcus epidermidis* ATCC 12228) were tested independently by two different technologists using different batches of reagents and antibiotics. The same instrument was used for the ATP assay.

Effect of Varying Inoculum Size on Microbial Sensitivity Testing by the ATP Index

The effect of varying the initial bacterial inoculum size, namely 5×10^4 cfu/ml and 5×10^7 cfu/ml, was studied and compared to the standard inoculum (1×10^6 cfu/ml). In order to perform the ATP assay with the smallest inoculum (5×10^4 cfu/ml), the procedure was modified as follows to give the increased sensitivity required.

1. Place a 5.0-ml sample of the broth culture in a sterile polypropylene tube (17 X 100 mm).
2. Add 1.0 ml of Apy-Ca-Tx (10 mg apyrase/ml) and vortex.
3. Centrifuge tube for 15 minutes at 10,400 RCF X G at 20°C.
4. Decant and invert on filter paper for 5 minutes.
5. Add 0.2 ml of 0.0625 N nitric acid to the precipitate. Vortex and allow to stand for 5 minutes.
6. Add 0.2 ml of sterile, deionized water, and vortex.
7. Assay 0.1 ml of this reaction mixture as in the previous method, except the photometer is standardized to 1.0×10^8 fg/ml of original sample by adding 0.05 ml of a freshly-thawed ATP standard (0.1 µg/ml) to the final reaction volume of a blank tube (sterile broth).

β-Lactamase Activity

The β-lactamase activity of the *S. aureus* strains tested was determined by the procedure of Foley and Perret.

RESULTS

An overall comparison of the results obtained by the ATP index and agar diffusion sensitivity testing is shown in table A-1. Of the 83 (10 percent) instances of disagreement, most (75 percent) were major, that is, false-resistance (46 of 63) or false-sensitivity (17 of 63). One-quarter of the disagreements were minor, that is intermediate by agar diffusion and either sensitive (11 of 20) or resistant (9 of 20) by the ATP index. The reasons for disagreement were studied in detail.

Agreement by Antibiotic

Ampicillin

Twelve of the 13 disagreements were with *Proteus* species (mainly *P. mirabilis*) which appeared falsely resistant to this antibiotic. The reason for this discrepancy is shown in figure A-1. The fall in bacterial ATP content which coincides with lysis of the organism by ampicillin (a cell wall-active or β-lactam-antibiotic) takes at least 3 hours after exposure to the drug, and earlier determination of drug-effect would predictably show false resistance.

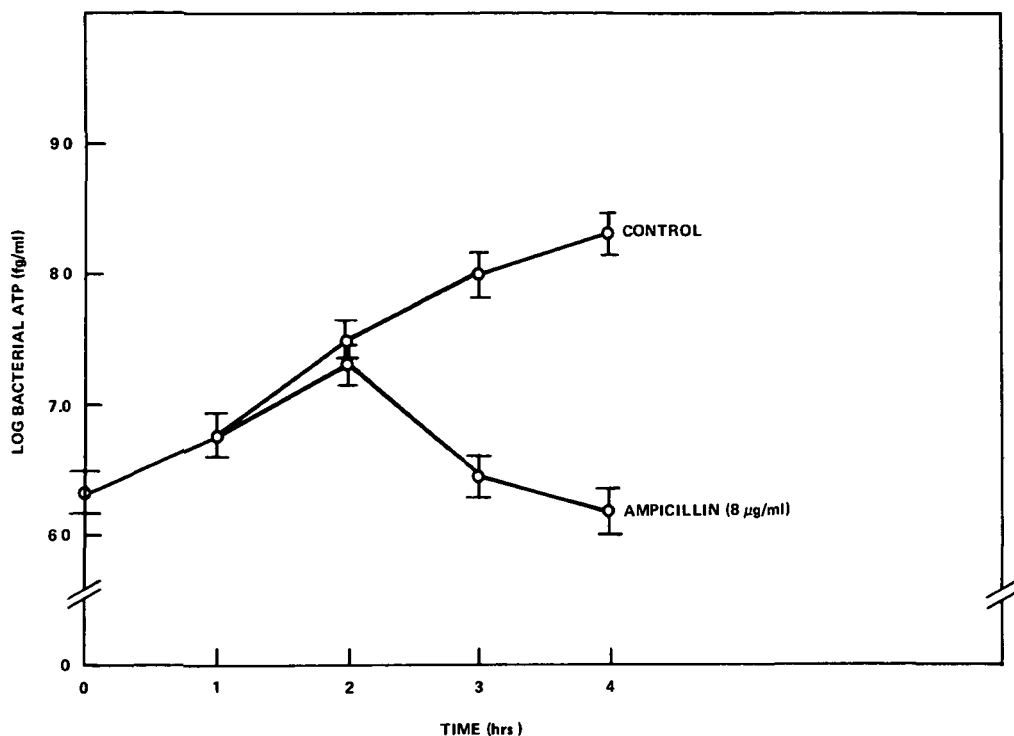


Figure A-1 Change in bacterial ATP when *P. mirabilis* (9 strains) were grown in the presence of ampicillin (8 µg/ml in TSB).

Penicillin G

The 10 strains that were in disagreement were confined to *P. mirabilis*, which appeared falsely-resistant to this antibiotic. This discrepancy is due to a similar delay in the lysis of *P. mirabilis* strains by penicillin G as was demonstrated for ampicillin.

The determination of *S. aureus* susceptibility to penicillin G presents unique difficulties. Penicillinase-producing *S. aureus* frequently appears sensitive to penicillin at 8 µg/ml after only a few hours of growth because there is insufficient time for penicillinase induction to take place. This problem can partly be overcome by using a much lower concentration of penicillin G, such as 0.075 µg/ml, which gives agreement in 17 of 18 instances (table A-2).

Carbenicillin

Only *E. coli*, *Proteus*, and *P. aeruginosa* strains were studied for susceptibility to this drug because these are the only genera for which interpretive zone standards are available. False-resistance was a frequent problem with *Pseudomonas* strains. This discrepancy is due to the fact that lysis of most *Pseudomonas* strains by carbenicillin takes longer than 6 hours (figure A-2).

Table A-2
Comparison of *S. aureus* Sensitivity to Penicillin G by Agar
Diffusion, Penicillinase Production, and the ATP Index at
Varying Concentrations of Penicillin

Strain	Agar Diffusion Zone Diameter	Interpretation	Penicillinase Production	ATP Index Penicillin G					
				0.05 g	INT	0.075	INT	0.10	INT
ATCC 25923.	32.5	S	-	+0.29	R	+0.04	S	+0.08	S
091	40	S	-	0.00	S	-0.13	S	-0.22	S
315	37	S	-	+0.12	S	0.01	S	+0.02	S
352	38.5	S	-	+0.22	S	+0.14	S	+0.07	S
511	37	S	-	+0.58	R	+0.57	R	+0.42	R
280	43	S	-	+0.31	R	+0.19	S	+0.05	S
187	18	R	+	+0.70	R	+0.85	R	+0.40	R
917	18.5	R	+	+1.00	R	+1.00	R	+0.92	R
752	20	R	+	+0.66	R	+0.53	R	+0.28	R
972	19	R	+	+0.94	R	+0.99	R	+0.24	R
801	17	R	+	+0.99	R	+0.94	R	+0.93	R
593	18	R	+	+0.99	R	+0.98	R	+0.92	R
896	22	I	+	+0.87	R	+0.62	R	+0.75	R
697	24.5	I	+	+0.60	R	+0.29	R	+0.14	S
565	25	I	+	+0.49	R	+0.38	R	+0.04	S
563	24	I	+	+0.36	R	+0.33	R	+0.07	S
581	23	I	+	+0.84	R	+0.71	R	+0.45	R
580	26.5	I	+	+0.71	R	+0.69	R	+0.28	R

Cephalothin

There were only four discrepancies with this drug, three instances of false-resistance with *P. mirabilis*, again reflecting the delayed time-to-lysis of these strains by cell wall-active β -lactam-antibiotics. The one instance of false sensitivity of an *Enterobacter* strain illustrates the uncommon instance, where, despite an apparent zone of inhibition in the 'susceptible' range by agar diffusion, several colonies are seen within the zone. Provided purity of strain can be confirmed, this must be interpreted as resistance, a fact confirmed by MIC determinations. A rapid susceptibility technique is likely to report these as falsely-sensitive, because the majority of the bacterial population in the inoculum is, in fact, susceptible to the drug.

Tetracycline

There were seven disagreements, but only two of these were major with no particular pattern.

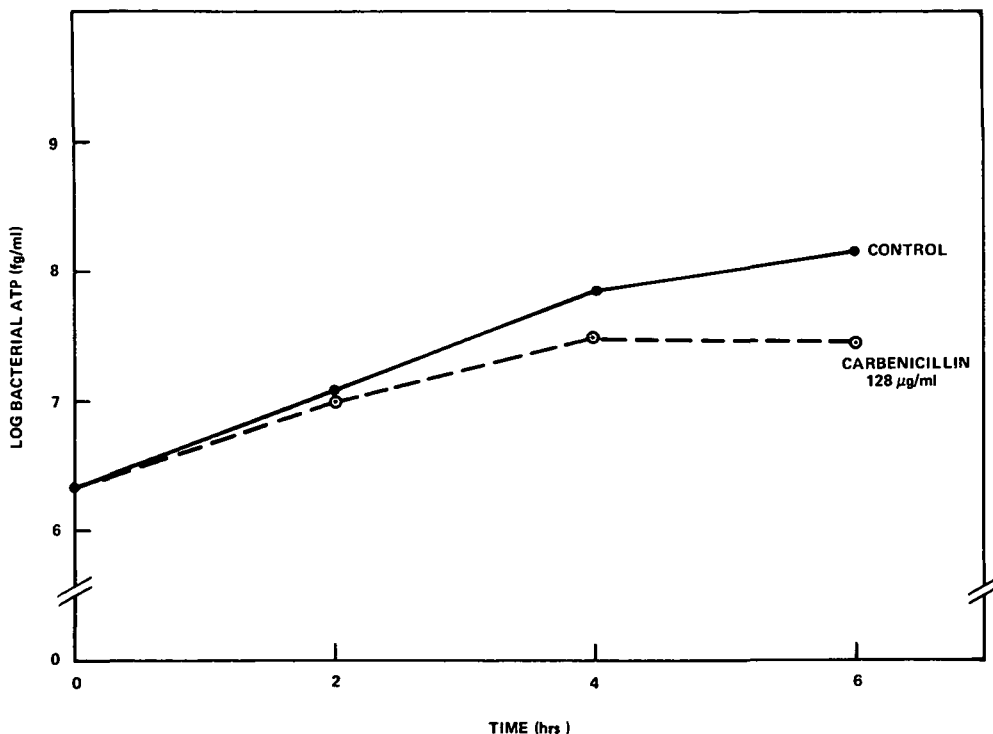


Figure A-2 Change in bacterial ATP when *Pseudomonas* were grown in the presence of Carbenicillin (128 µg/ml).

Erythromycin

There were no major disagreements with this antibiotic. The four minor disagreements were confined to *enterococci* which were sensitive by the ATP index and intermediate by disc.

Clindamycin

All nine instances of disagreement were with *enterococci* that were sensitive by the ATP index and resistant by disc. The reason for this discrepancy is shown on figure A-3. Considerably subinhibitory concentrations (that is, much less than the MIC) of clindamycin inhibit ATP synthesis during the first two hours, but thereafter ATP synthesis (and bacterial multiplication) proceeds at a rate similar to that of the control. Thus, there is an apparent lag before the ultimate resistance of the organism becomes apparent.

Gentamicin

There was complete agreement between the two methods with this aminoglycoside antibiotic.

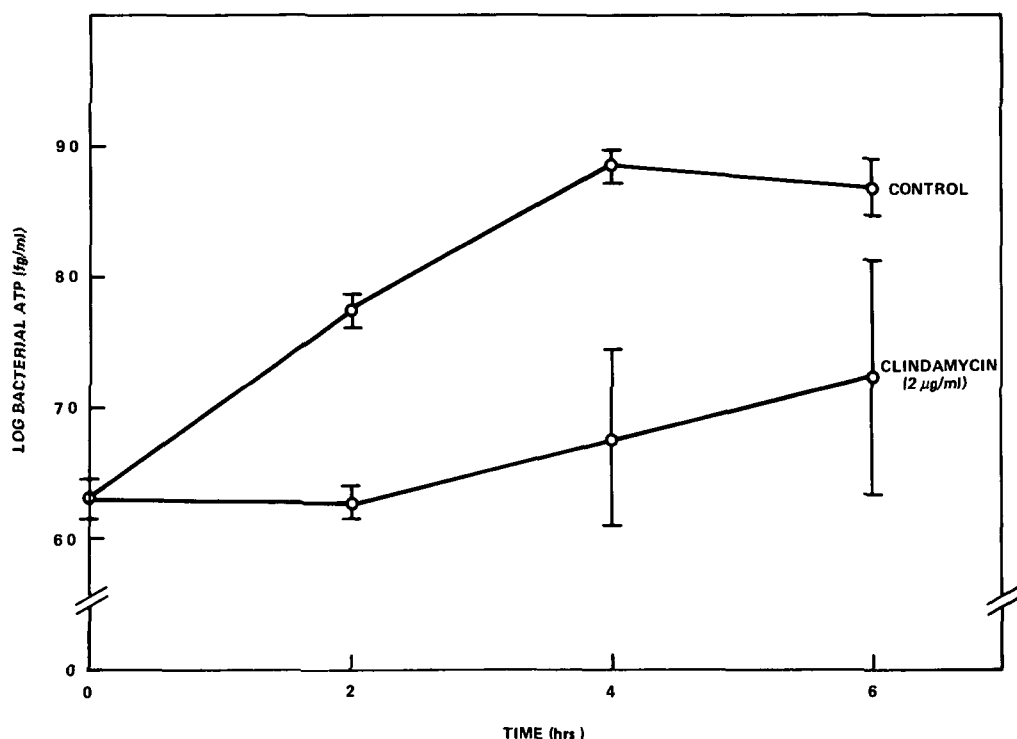


Figure A-3. Change in bacterial ATP when *enterococcus* (10 strains) were grown in the presence of clindamycin (2 µg/ml).

Nitrofurantoin

There were 19 disagreements, 6 minor and 13 major. Twelve of the major disagreements were instances of false-resistance to nitrofurantoin by *staphylococci* and *enterococci*. The ATP index in all these instances was very close to the breakpoint value of +0.25. When this problem was studied in more detail (figure A-4), the inhibition of *S. aureus* by nitrofurantoin was not apparent until after 1 hour of incubation. Due to the relatively slower growth of the control organism in comparison to gram negative organisms, the requisite index for sensitivity cannot quite be achieved even in 3 hours. This problem could be solved by applying a different interpretive criteria to this drug, namely a breakpoint ATP index of +0.35.

Colistin

There were only two disagreements, both instances of false-sensitivity to *Enterobacter-Serratia* strains. These again were instances where there was an apparent zone of inhibition in the sensitive range by agar diffusion, but several colonies were seen within the zone, necessitating an interpretation of resistance.

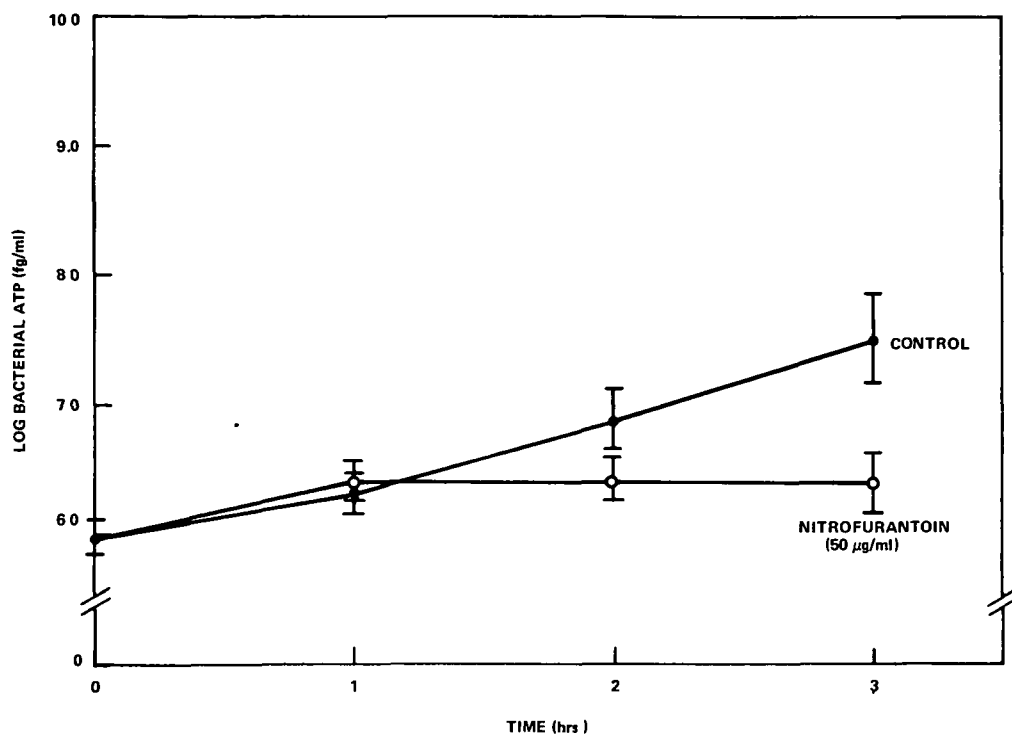


Figure A-4. Change in bacterial ATP when *S. aureus* (10 strains) were grown in the presence of nitrofurantoin (50 µg/ml).

Chloramphenicol

There were seven discrepancies with this antibiotic (five major, two minor) distributed among several species with no particular pattern of disagreement.

Sulfisoxazole

Rapid sensitivity testing to this agent appears impossible by this technique. The ATP index consistently gave false-resistant results. As shown in figure A-5, there was no sulfonamide effect measurable after 3 hours of log phase growth using an initial inoculum of 5×10^4 cfu/ml.

Nalidixic Acid

The ATP index consistently gives false-resistant results with this agent as well (figure A-6).

Reproducibility Studies

Table A-3 summarizes the results from each technologist. Each one achieved the same degree of overall agreement with the agar diffusion method. This agreement was similar to that achieved by the ATP index in general (90 percent). There is a 94-percent agreement between the technologists.

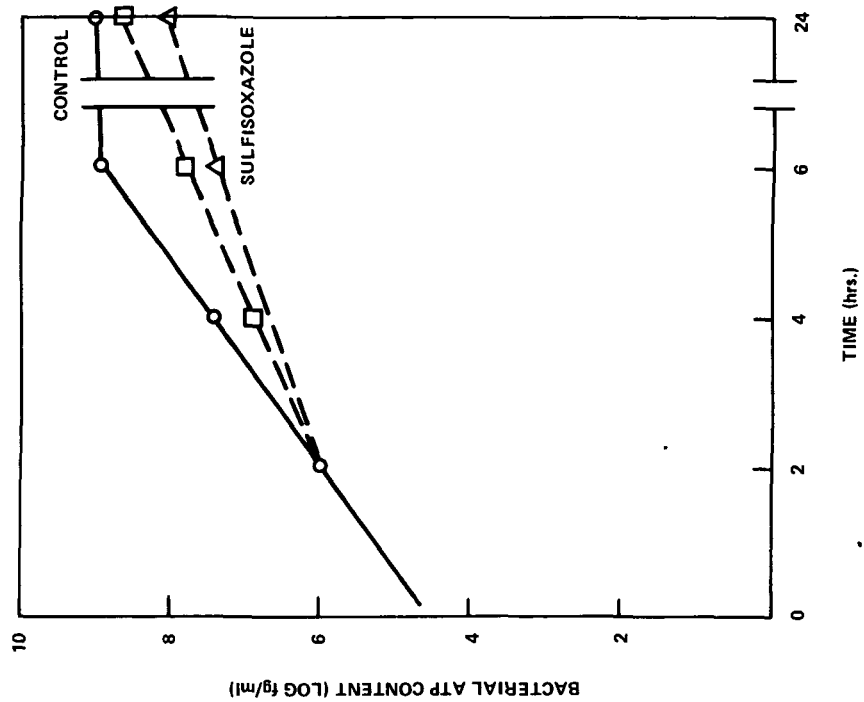
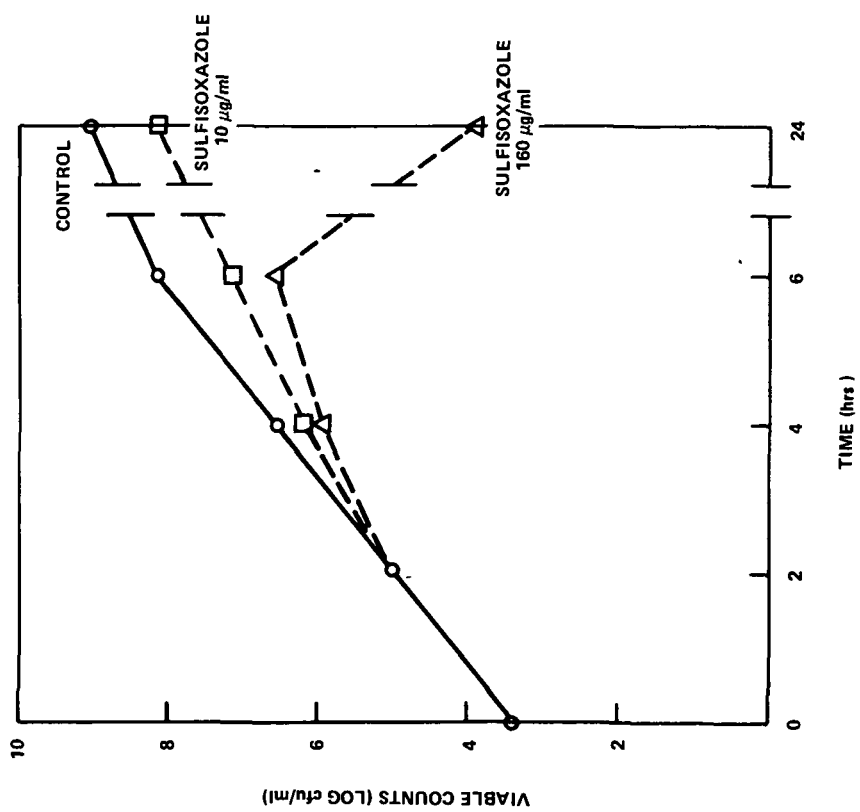


Figure A-5. Change in bacterial ATP and colony counts when *E. coli* ATCC 25922 were grown in the presence of sulfisoxazole (10 µg/ml and 160 µg/ml).

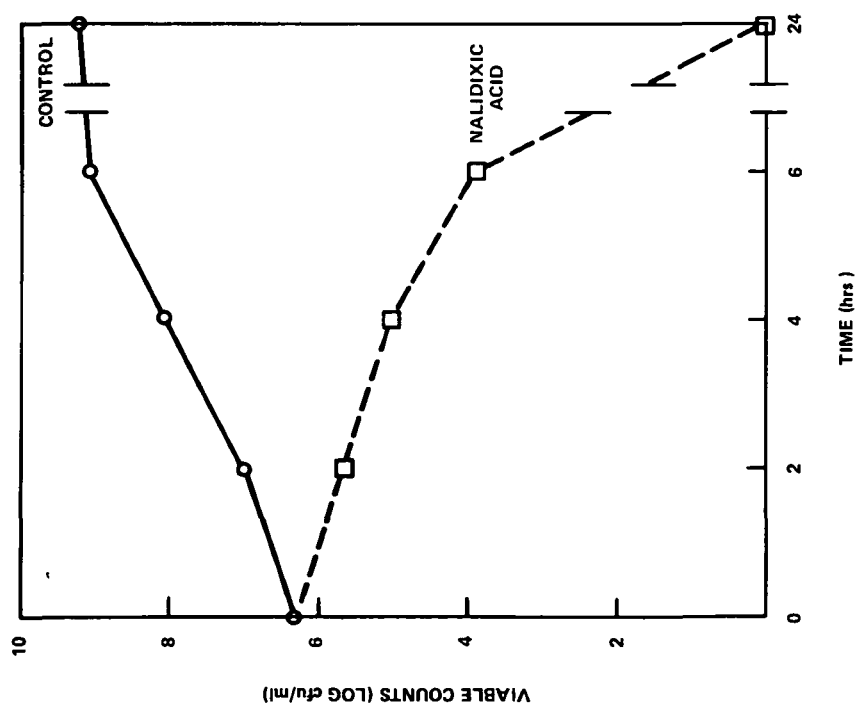
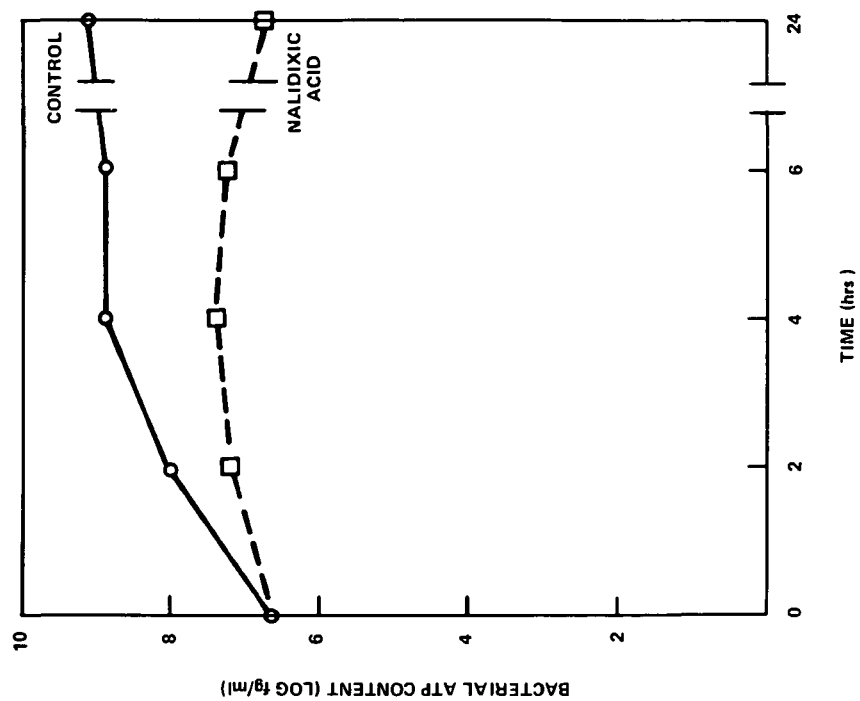


Figure A-6. Change in bacterial ATP and colony counts when *E. coli* were grown in the presence of nalidixic acid (12.5 µg/ml).

Table A-3
Reproducibility of the ATP Index When
Performed by Different Technologists

Antibiotic	No. Strains Tested	Major disagreements with Agar Diffusion		Disagreements Between Tech-1 and Tech-2
		Tech-1	Tech-2	
Ampicillin	8	2	3	1
Penicillin G	8	0	0	0
Carbenicillin	4	0	0	0
Cephalothin	10	0	0	0
Tetracycline	10	0	0	1
Erythromycin	10	0	0	0
Clindamycin	10	0	0	0
Gentamicin	10	1	0	1
Nitrofurantoin	10	2	2	1
Colistin	10	1	0	1
Chloramphenicol	10	2	3	1
TOTAL	100	8	8	6

Effect of Varying Inoculum Size

Although there was considerable variation in the absolute value of the ATP index (especially when negative values were obtained), there was 100-percent correlation with the standard 1×10^6 cfu/ml inoculum when an inoculum of 5×10^4 cfu/ml was used. Valid data could not be obtained for the *S. aureus* strain used (ATCC 25923) because there was no significant increment in the ATP content of the control organism during the 2.5-hour period of incubation. Using an inoculum of 5×10^7 cfu/ml, there were five instances of disagreement with the method using the standard inoculum: the *K. aerogenes* and *P. mirabilis* strains resistant to nitrofurantoin appear falsely-sensitive to this drug; and the *E. coli* and *Klebsiella* strains sensitive to colistin appear falsely-resistant. Valid data could not be obtained for the *P. aeruginosa* strain used because there was no significant increment in the ATP content of the control organism during the 2.5-hour period of incubation.

DISCUSSION

The system for determining microbial susceptibility to antibiotics that we have developed using the luciferase assay for ATP gives a 90-percent correlation with existing rapid methods that have undergone extensive laboratory evaluation.

The 2.5 hours of incubation required to achieve the 90-percent correlation seem to be the minimum duration necessary for adequate multiplication of the control organism (three to five doubling times) and to demonstrate drug effect at concentrations commonly achieved in clinical practice. Extending the incubation period by 1 hour would clearly improve the correlation (for example, β -lactam antibiotics against *P. mirabilis*). There are infrequent instances where the increment in bacterial ATP during the usual incubation period is insufficient to allow any valid conclusions to be drawn about antibiotic effect. At the standard inoculum size, this problem is virtually limited to *S. aureus* strains. Due to its infrequent occurrence, we have not yet evaluated the effect of adding 0.2 percent KNO_3 to the medium.

The inoculum size for most microbial susceptibility tests is carefully standardized, particularly for the agar-diffusion method. Although we have used a standard inoculum of 1×10^6 cfu/ml, evaluation of a smaller inoculum (5×10^4 cfu/ml) gives equally good results except for the failure of the *S. aureus* strain tested to demonstrate a significant increment in ATP content during the incubation period of the control. Less reproducible results were obtained with the larger inoculum of 5×10^7 cfu/ml.

The interpretive criteria that has been tentatively adopted for the ATP index may yet be subject to modification as further experience is gained with this method. It may not be feasible to establish a single ATP index for all antimicrobials, as has already been noted with nitrofurantoin. We are unable at this time to define an "intermediate" category of sensitivity.

The major cause for disagreement between the ATP index and agar diffusion is the mode of action of the antimicrobial agent. This is particularly evident of cell wall-active β -lactam-antibiotics on gram-negative bacteria, where one-half of all the major disagreements were seen, including all instances of false-resistance. When a β -lactam-antibiotic is added to a sensitive gram-negative bacterium in the logarithmic phase of growth, the bacterium undergoes a series of morphological changes from filamentous to bulbous or "bow-tie" forms to spheroblasts before finally undergoing osmotic lysis. These changes are related to drug-induced alterations in the cell-wall structure and may be dependent on the relative inhibitory effect of β -lactam antibiotics on two different enzymes: an endopeptidase concerned with cell division and a glycosidase concerned with cell growth. The rate at which these changes occur are dependent on the specific drug, on its concentration, on the type and rate of production of β -lactamase by the bacterium, and on the stability of the drug to β -lactamase. In addition, the time to lysis is dependent on the osmolality of the medium (being more rapid at lower osmolalities) and the osmotic susceptibility of the species with damaged cell walls (*E. coli* being more susceptible than *P. mirabilis*) (Greenwood and O'Grady, 1969). Continuous turbidometric recordings of these events show increasing opacification of the broth cultures (indicating increasing bacterial cell mass despite absence of cell division) for varying lengths of time after the addition of the drug until lysis occurs. Bacterial protein synthesis is not inhibited by this class of drugs.

When bacterial ATP content is used as a measure of β -lactam antibiotic effect on log phase cultures, the drop in ATP (indicating sensitivity) occurs at the time of lysis. A prolonged time to achieve lysis was seen with penicillin G and ampicillin against *P. mirabilis* (figure A-1) and carbenicillin against *P. aeruginosa* (figure A-2). These findings are consistent with those of other investigators using a turbidometric index of cell lysis. This phenomenon may make it necessary to extend the minimum period of incubation to one which will encompass the time to lysis of such commonly encountered species as *P. mirabilis*.

Sulfonamides act as competitive inhibitors of p-amino benzoic acid in the biosynthetic reaction to form dihydrofolate in bacteria. *In vitro* sensitivity to sulfonamides has always presented special difficulties because of: (1) the presence in many microbiological media of substances that inhibit the antibacterial action of sulfonamides (this can be neutralized by the addition of lysed horse blood) and (2) the necessity of using a very small inoculum of organisms since bacteria must undergo many generations before any drug effect becomes apparent. The latter point effectively precludes 'rapid' measurement of drug effect; as would be expected, the ATP index consistently gave false-resistant results (figure A-5).

The ATP index consistently gave false-resistant results with nalidixic acid (figure A-6). This was not surprising in view of the fact that nalidixic acid is known to exert its antibacterial effect primarily by interfering with synthesis of deoxyribonucleic acid (DNA). Formation of ribonucleic acid (RNA), protein, and lipid are relatively undisturbed so that synthesis of ATP proceeds for a substantial period of time. Nalidixic acid has little or no effect on respiration with glucose as substrate.

The major advantage of the ATP system is that the assay can be made specific for bacterial ATP. Both the Technicon Automated Antibiotic Susceptibility (TAAS) System and the Autobac 1tm system use an optical cell counting instrument that necessitates the use of specially prepared "optically clean" liquid media. Our laboratory is currently developing a method by which rapid antimicrobial susceptibility can be applied directly to organisms in urine without prior bacterial isolation or subculture. This application requires a technique of measuring bacterial growth that is specific for bacteria and not interfered with by particular matter that is found in biological fluids such as urine.

The procedure described is compatible with automation since automated systems will be necessary to perform the large number of assays involved.

APPENDIX B
PROCEDURE AND EXPERIMENTS

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APPENDIX B

PROCEDURE AND EXPERIMENTS

PROCEDURE 1: SCREENING—LONG CENTRIFUGATION

1. Centrifuge a .35-ml urine sample at 8,000 RCF $\times$ G for 15 minutes at 4°C.
2. Reconstitute pellet with 1.75 ml TSB.
Vortex.
Pipette 0.5 ml into a 17-x 100-mm polypropylene tube.
Store remaining 1.25 ml in refrigerator.
3. Add 1.0 ml apyrase (10 mg/ml 0.03 M CaCl_2 -0.6 percent TX) to 0.5 ml reconstituted urine sample.
Vortex.
Add 4.5 ml 0.9 percent N saline.
Add 0.02 ml 6.0 percent TX.
Vortex.
Wait 15 minutes.
4. Add 1.0 ml 0.25 M malic acid.
Vortex.
5. Centrifuge at 8,000 RCF $\times$ G for 15 minutes at 4°C.
Invert and drain for 5 minutes.
6. Add 0.2 ml 0.1 N HNO_3 .
Vortex.
Wait 5 minutes.
7. Add 0.2 ml sterile distilled H_2O .
Vortex.
8. Inject 0.1 ml sample into 0.1 ml luciferase (prepared one hour before by adding 1.5 ml 0.25 M TRIS, 0.01 M MgSO_4 , pH 8.2, to one vial of DuPont luciferase powder).
Read biometer for ATP light units.

PROCEDURE 2: DETERMINATION OF ANTIBIOTIC SENSITIVITIES

Nonconcentrated Procedure Without Malic Acid

1. Retrieve 1.25 ml sample from refrigerator.
Dilute with TSB to achieve appropriate ATP level.
2. Preincubate 0.5 hour at 37°C.

3. Set up 17-x 100-mm polypropylene tubes to contain as follows:
 - A_0 = 0.45 ml sample +0.05 ml sterile distilled H_2O
 - $A_{2.5}$ = 0.45 ml sample +0.05 ml sterile distilled H_2O
 - B_{1-10} = 0.45 ml sample +0.05 ml antibiotic (aliquots previously frozen in tubes and brought to room temperature)
 - A_0 = 0.40 ml sample +0.10 ml TSB
 - $A_{2.5}$ = 0.40 ml sample +0.10 ml TSB
 - B_{11} = 0.40 ml sample +0.10 ml nitrofurantoin
4. Incubate all except the A_0 's at 37°C for 2.5 hours.
5. Add 0.1 ml apyrase (that remaining from Procedure 1) to A_0 's.
Vortex.
Wait 15 minutes.
6. Add 0.1 ml 1.5 N HNO_3 to A_0 's.
Vortex.
Wait 5 minutes.
7. Add 4.3 ml sterile distilled H_2O to A_0 's.
Vortex.
Hold at room temperature.
8. At end of 2.5-hour incubation period, remove tubes from incubator.
Add 0.1 ml apyrase (10 mg/ml 0.03 M $CaCl_2$ -0.6 percent TX, prepared anew).
Vortex.
Wait 5 minutes.
9. Add 0.1 ml 1.5 N HNO_3 .
Vortex.
Wait 15 minutes.
10. Add 4.3 ml sterile distilled H_2O .
11. Assay all tubes by injecting a 0.1-ml sample (mixing sample with syringe) into 0.1 ml luciferase (prepared one hour before by adding 1.5 ml 0.25 M TRIS, 0.01 M $MgSO_4$, pH 8.2, to one vial of DuPont luciferase powder).
Read biometer for ATP light units.

EXPERIMENT 1: STABILITY OF THE A_0 WITH *E. COLI*

Purpose

In order to determine antibiotic sensitivities at the end of a 2.5-hour incubation period, the level of ATP at time zero must be established. Consistency and a more efficient use of luciferase would be achieved if the A_0 could be assayed 2.5 hours later with those samples containing antibiotic. The purpose of this experiment was to determine how to store the A_0 during the given incubation period so that the level of ATP would not change significantly.

Procedure

1. Inoculate 20 ml of urine with an overnight growth of *E. coli* in TSB to achieve 10^7 organisms/ml.
2. Centrifuge at 8,000 RCF $\times$ G for 15 minutes at 4°C.
3. Reconstitute pellet in 20 ml TSB.
4. Preincubate for 0.5 hour at 37°C.
5. Pipette 0.5-ml aliquots of infected broth into 7 tubes and prepare each as follows:
 - a. Refrigerate U_1 for 2.5 hours.
 - b. Hold U_2 at room temperature for 2.5 hours.
 - c. Incubate U_3 at 37°C for 2.5 hours.
 - d. Extract ATP from bacteria in U_4 , U_5 , and U_6 , first by adding 0.1 ml 10 mg apyrase/ml 0.03 M CaCl_2 - 0.6 percent TX (vortex and wait 15 min. for hydrolysis of soluble ATP), then adding 0.1 ml 1.5 N HNO_3 (vortex and wait 5 minutes. for extraction of bacterial ATP), and finally diluting with 4.3 ml H_2O (vortex):
 1. Freeze U_4 for 2.5 hours.
 2. Refrigerate U_5 for 2.5 hours.
 3. Hold U_6 at room temperature for 2.5 hours.
 - e. Extract ATP from bacteria in the seventh sample and from 0.5 ml of TSB (as described above), and assay by injecting 0.1 ml final processed sample into 0.1 ml luciferase in 0.25 M TRIS, 0.01 M MgSO_4 , pH 8.2. These samples will serve as the A_0 and the blank (U_{control}).
6. At the end of the 2.5-hour period, bring all samples to room temperature, extract bacterial ATP (as described above) in U_1 , U_2 , U_3 , and assay U_1 , U_2 , U_3 , U_4 , U_5 , and U_6 by injecting into luciferase.

The results of this experiment are shown in table B-1.

Conclusions

If the A_0 , containing intact bacteria, is stored at room temperature or 37°C, for 2.5 hours, bacterial ATP increases significantly. Refrigeration seems to kill a small proportion of the organisms (or to decrease their ATP content).

If the bacteria are lysed by the addition of HNO_3 and the sample is diluted before storing, no change in the ATP level occurs whether the sample is stored in the freezer, refrigerator, or left at room temperature. This conclusion is restricted to a 2.5-hour storage period of the A_0 and to a urine inoculated with *E. coli*.

Table B-1
Results of Various Treatments of Sample before Assay

Sample	Treatment Before Assay	Light Units
U_{control}	assayed immediately	2.94×10^5
A_0	assayed immediately	4.50×10^7
U_1	refrigerated, extracted, diluted	3.67×10^7
U_2	room temperature, extracted, diluted	3.03×10^8
U_3	37°C, extracted, diluted	8.86×10^8
U_4	extracted, diluted, frozen	4.55×10^7
U_5	extracted, diluted, refrigerated	4.51×10^7
U_6	extracted, diluted, room temperature	4.58×10^7

EXPERIMENT 2: STABILITY OF THE A_0 WITH OTHER BACTERIAL STRAINS

Purpose

Experiment 1 demonstrated stability of bacterial ATP in the A_0 of a urine sample infected with *E. coli* when the ATP is extracted, diluted, and stored for 2.5 hours at room temperature or lower. This experiment reexamines the problem using urines inoculated with organisms from reference strains of *E. coli*, *Klebsiella*, *Enterobacter*, *Enterococcus*, *S. aureus*, *S. epidermidis*, *P. mirabilis*, and *Pseudomonas* and a storage period extended to 5 hours.

Procedure

1. Inoculate 4.5 ml of urine with an overnight growth of a reference strain organism in TSB to achieve 10^7 organisms/ml.
2. Centrifuge at 8,000 RCF $\times$ G for 15 minutes at 4°C.
3. Reconstitute pellet in 5.0 ml TSB; preincubate 0.5 hour.
4. Pipette 0.5-ml aliquots into four tubes for each reference strain:
 - a. Refrigerate tubes b for 5 hours.
 - b. Incubate tubes c for 5 hours at 37°C.
 - c. Add apyrase, HNO_3 , and H_2O to tubes a and d.
 1. Hold tubes a at room temperature for 5 hours.
 2. Assay tubes d to serve as true A_0 's.

5. After 5 hours bring tubes b and c to room temperature, and add apyrase, HNO_3 , and H_2O .
6. Assay tubes a, b, and c.

Results

Bacterial ATP in most of those samples stored at refrigerated or higher temperatures with bacterial cells intact varied considerably from the original A_0 level: summing ATP levels of similar magnitude indicates a trend of decay in tubes b and a trend of growth in tubes c. Good correlation is shown between the A_0 and those samples from which the bacterial ATP has been extracted, diluted, and held at room temperature for 5 hours: summing ATP levels of similar magnitude indicates a trend of stability in tubes a.

Although the A_0 's for *S. aureus* and *S. epidermidis* are high (perhaps the original overnight growths were not sufficiently diluted), the relationships between tubes a, b, c, and d are still valid.

Conclusion

Once the bacteria in a urine sample are lysed by the addition of HNO_3 and then diluted in H_2O , the A_0 may be stored for as long as 5 hours without sacrificing accuracy. This conclusion holds for all eight reference strains (see table B-2).

Table B-2
Results of Experiment 2: Stability of A_0 After Extraction,
Dilution, and a 5-hour Incubation Period

	A_0 (light units)	Tube a Room Temperature	Tube b Refrigerated	Tube c 37°C
U ₁ <i>Pseudomonas</i>	2.75×10^7	2.89×10^7	2.59×10^7	9.49×10^8
U ₂ <i>Enterococcus</i>	7.53×10^7	7.52×10^7	4.77×10^7	7.65×10^8
U ₃ <i>P. mirabilis</i>	4.05×10^7	4.67×10^7	2.91×10^7	2.72×10^9
U ₄ <i>S. epidermidis</i>	3.10×10^8	3.42×10^8	3.72×10^8	2.09×10^9
U ₅ <i>S. aureus</i>	2.15×10^9	2.55×10^9	1.87×10^9	8.35×10^8
U ₆ <i>Klebsiella</i>	9.76×10^7	8.76×10^7	6.16×10^7	7.56×10^8
U ₇ <i>Enterobacter</i>	4.21×10^7	3.96×10^7	3.23×10^7	1.38×10^9
U ₈ <i>E. coli</i>	5.20×10^7	5.70×10^7	5.49×10^7	1.19×10^9
Totals	3.35×10^8	3.35×10^8	2.52×10^8	7.76×10^9
U control (uninfected)	1.90×10^5	3.04×10^5	5.65×10^5	3.49×10^5

EXPERIMENT 3: KANAMYCIN AND STREPTOMYCIN

Purpose

The purpose of this experiment is to determine the lowest concentration of kanamycin and streptomycin that will give sensitivities at 2.5 hours in agreement with Kirby-Bauer results for broths infected with *Klebsiella*, *Enterobacter*, *Enterococcus*, *S. aureus*, *S. epidermidis*, *P. mirabilis*, or *Pseudomonas*. The lowest possible antibiotic concentration is preferred in the luciferase assay because it offers a greater degree of discrimination between sensitivity and resistance, and because the more highly concentrated antibiotic might enhance the occurrence of false sensitivities.

Procedure

1. Inoculate 5 ml of TSB with an overnight growth of a reference organism in TSB to achieve 10^6 organisms/ml.
2. Preincubate 0.5 hour.
3. Pipette 0.45 ml aliquots of sample and add 0.05 ml of antibiotic to achieve these final concentrations:

kanamycin (K_1) = 25 $\mu\text{g/ml}$

kanamycin (K_2) = 15 $\mu\text{g/ml}$

kanamycin (K_3) = 6 $\mu\text{g/ml}$

streptomycin (S_1) = 15 $\mu\text{g/ml}$

streptomycin (S_2) = 10 $\mu\text{g/ml}$

streptomycin (S_3) = 6 $\mu\text{g/ml}$

4. Prepare A_0 and $A_{2.5}$ controls with sterile distilled H_2O instead of antibiotic.
5. Extract and assay A_0 immediately (as described in Appendix B, Procedure 2).
6. Incubate remaining samples at 37°C for 2.5 hours.
7. Extract and assay samples.

Results

1. No concentration of kanamycin gave results in agreement with Kirby-Bauer for *S. epidermidis*.
2. K_1 and K_2 gave 100-percent agreement with Kirby-Bauer for the other six organisms.
3. K_3 gave false-resistant results for two of those six organisms.

4. S_1 and S_2 gave 100-percent agreement with Kirby-Bauer for all seven organisms.
5. S_3 gave false-resistant results for two of the seven organisms.

Conclusions

The lowest concentration of kanamycin that will give sensitivities at 2.5 hours that agree with Kirby-Bauer results for six of the organisms tested (no concentration of the antibiotic was shown to work in the case of *S. epidermidis*) is 15 $\mu\text{g/ml}$.

The lowest concentration of streptomycin that will give accurate sensitivities for all seven organisms tested is 10 $\mu\text{g/ml}$ (see table B-3).

EXPERIMENT 4: LONG CENTRIFUGATION VERSUS NONCONCENTRATED PROCEDURE WITHOUT MALIC ACID

Purpose

The Long Centrifugation procedure, used to detect and quantitate bacterial ATP in a urine sample, is described in Procedure 1 of this appendix. This determination is necessary in order to prepare an inoculum of about 10^6 bacteria/ml to initiate subsequent antibiotic susceptibility testing also by ATP assays. Assays in the susceptibility test, however, involve a different, less specific assay, the nonconcentrated procedure without malic acid. Before an inoculum can be prepared for susceptibility testing, the disparity between values obtained by the two procedures for a given sample must be taken into account. This experiment seeks to establish that disparity by inoculating urines with given levels of bacteria and comparing ATP levels obtained by each method.

Procedure

1. Pipette 12 ml of urine into three tubes.
2. Inoculate tube a with 3×10^6 bacteria/ml
tube b with 1×10^7 bacteria/ml
tube c with 1×10^8 bacteria/ml
(diluted from overnight growths of four reference strains in TSB).
3. Centrifuge at 8,000 RCF $\times$ G for 15 minutes at 4°C.
4. Reconstitute with 1.2 ml TSB.
5. From tubes a, b, and c pipette 1.0 ml for Long Centrifugation procedure.
 - a. Add 1.0 ml of apyrase (10 mg/ml 0.03 M CaCl_2 - 0.6 percent TX), 4.5 ml 0.9 percent Normal saline, and 0.02 ml of 6.0 percent TX to 1.0 ml sample. Vortex and wait 15 minutes.
 - b. Add 1.0 ml 0.25 M malic acid, vortex, and centrifuge at 8,000 RCF $\times$ G for 15 minutes at 4°C. Invert and drain 5 minutes.

Table B-3
Results for Experiment 3: Sensitivity of Seven Organisms to Three
Concentrations of Kanamycin and Streptomycin

	ATP	Index	Index Interpretation	KB Interpretation	ATP	Index	Index Interpretation	KB Interpretation
<i>Klebsiella</i>				2	<i>P. mirabilis</i>			
A ₀	6.93×10^7				4.57×10^7			
A _{2.5}	9.62×10^8				5.90×10^8			
K ₁	1.69×10^7	-0.06	S	S	2.40×10^7	-0.04	S	S
K ₂	1.57×10^7	-0.06	S	S	3.37×10^7	-0.02	S	S
K ₃	1.89×10^8	-0.13	R	S	4.23×10^7	-0.01	S	S
A ₀	7.60×10^7				4.33×10^7			
A _{2.5}	1.36×10^9				6.26×10^8			
S ₁	1.33×10^8	+0.04	S	I	2.60×10^7	-0.03	S	S
S ₂	4.23×10^8	+0.27	R	I	3.38×10^7	-0.02	S	S
S ₃	8.07×10^8	+0.57	R	I	9.52×10^7	+0.09	R	S
<i>Enterobacter</i>					<i>Enterococcus</i>			
A ₀	3.54×10^7				7.54×10^7			
A _{2.5}	5.60×10^8				1.63×10^7			
K ₁	1.40×10^6	-0.06	S	S	1.74×10^9	+1.07	R	R
K ₂	2.94×10^6	-0.06	S	S	1.67×10^9	+1.03	R	R
K ₃	8.95×10^7	+0.10	R	S	1.63×10^9	+1.00	R	R
A ₀	3.15×10^7				7.31×10^7			
A _{2.5}	5.23×10^8				1.79×10^9			
S ₁	3.79×10^6	-0.06	S	S	1.60×10^9	+0.89	R	R
S ₂	1.75×10^7	-0.03	S	S	1.67×10^9	+0.93	R	R
S ₃	9.47×10^7	+0.13	R	S	1.44×10^9	+0.80	R	R
<i>S. aureus</i>					<i>Pseudomonas</i>			
A ₀	1.77×10^6				7.33×10^5			
A _{2.5}	4.84×10^7				1.97×10^7			
K ₁	2.54×10^6	+0.02	S	S	8.27×10^6	+0.40	R	R
K ₂	2.33×10^6	+0.01	S	S	2.03×10^7	+1.03	R	R
K ₃	2.56×10^6	+0.02	S	S	2.72×10^7	+1.40	R	R
A ₀	1.84×10^6				8.06×10^5			
A _{2.5}	4.70×10^7				2.61×10^7			
S ₁	7.63×10^5	-0.02	S	S	2.00×10^7	+0.76	R	R
S ₂	6.83×10^5	-0.39	S	S	2.21×10^7	+0.84	R	R
S ₃	1.91×10^6	+0.00	S	S	1.75×10^7	+0.66	R	R

Table B-3 (Continued)

	ATP	Index	Index Interpretation	KB Interpretation	ATP	Index	Index Interpretation	KB Interpretation
<i>S. epidermidis</i>								
A ₀	8.35×10^5							
A _{2.5}	5.81×10^6							
K ₁	2.16×10^6	+0.27	R	S				
K ₂	2.26×10^6	+0.29	R	S				
K ₃	3.00×10^6	+0.44	R	S				
A ₀	7.79×10^5							
A _{2.5}	5.63×10^6							
S ₁	5.44×10^6	+0.96	R	R				
S ₂	5.17×10^6	+0.90	R	R				
S ₃	5.37×10^6	+0.95	R	R				

- c. Add 0.2 ml 0.1 N HNO_3 , vortex, and wait 5 minutes.
Dilute with 0.2 ml sterile, distilled H_2O , and vortex.
 - d. Assay by injecting 0.1 ml final sample into 0.1 ml luciferase in 0.25 M TRIS, 0.01 M MgSO_4 , pH 8.2.
6. From tubes a, b, and c, pipette 0.05 ml for nonconcentrated procedure without malic acid.
 - a. Add 0.45 ml of TSB.
 - b. Incubate 0.5 hour at 37°C .
 - c. Add 0.1 ml of apyrase (10 mg/ml 0.03 M CaCl_2 - 0.6 percent TX), vortex, and wait 15 minutes.
 - d. Add 0.1 ml 1.5 N HNO_3 , vortex, and wait 5 minutes.
Dilute with 4.3 ml sterile, distilled H_2O .
 - e. Assay by injecting a 0.1-ml sample into 0.1 ml same luciferase.

Results

A linear correlation is shown to exist between the ATP values obtained by the two procedures for each organism tested (see figure B-1 and table B-4). The average slope is approximately 40.

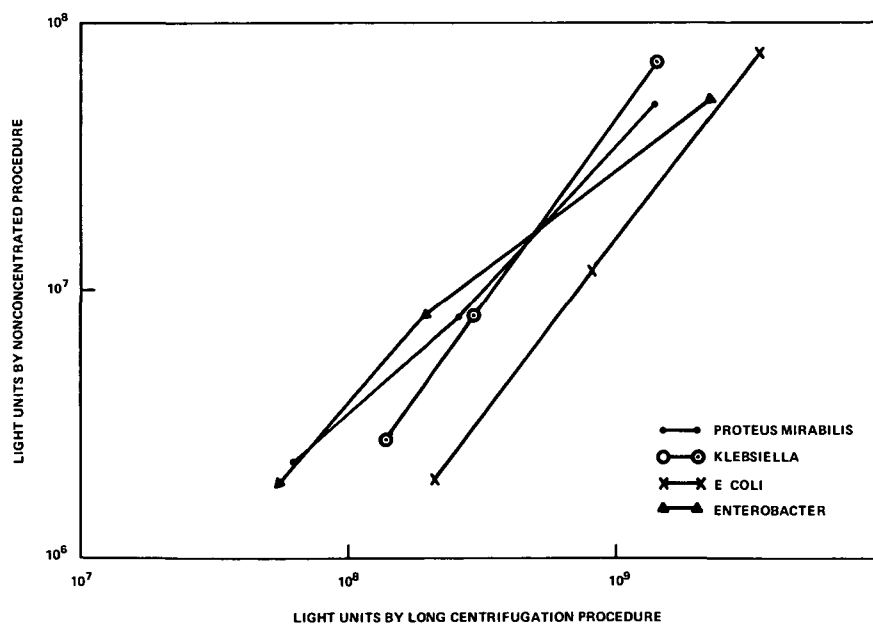


Figure B-1 Comparison of the Long Centrifugation procedure and the Nonconcentrated procedure.

Table B-4
Results for Experiment 4: Comparison of the Long Centrifugation
Procedure and the Nonconcentrated Procedure

Organism	Inoculum size (bacteria/ml)	Long Centrifugation Procedure (light units)	Nonconcentrated Procedure (light units)	Ratio	Average Ratio
<i>P. mirabilis</i>	3×10^5	6.05×10^7	2.27×10^6	26.65	29.12
	1×10^6	2.59×10^8	8.01×10^6	32.33	
	1×10^7	1.39×10^9	4.90×10^7	28.37	
<i>Klebsiella</i>	3×10^5	1.38×10^8	2.77×10^6	49.82	35.61
	1×10^6	2.93×10^8	8.04×10^6	36.44	
	1×10^7	1.44×10^9	7.00×10^7	20.57	
<i>E. coli</i>	3×10^5	2.09×10^8	1.94×10^6	107.73	72.74
	1×10^6	8.14×10^8	1.19×10^7	68.40	
	1×10^7	3.28×10^9	7.79×10^7	42.10	
<i>Enterobacter</i>	3×10^5	5.50×10^7	1.90×10^6	28.95	32.60
	1×10^6	1.95×10^8	8.13×10^6	23.40	
	1×10^7	2.30×10^9	5.06×10^7	45.45	
Controls	—	5.90×10^5	2.98×10^5	—	
	—	1.35×10^5	3.60×10^5	—	
	—	2.67×10^5	3.64×10^5	—	

Conclusions

To estimate bacterial ATP levels in light units that would be obtained by the nonconcentrated procedure without malic acid after a 30-minute preincubation period, divide the long centrifugation light-unit value by about 40. This denominator can be used to construct a dilution schedule (see table 5-4 on page 81) that will ensure the ATP light unit level of the A_0 to fall in the desired range of 1.0×10^5 to 1.0×10^7 light units on the biometer.

For example, if the long-centrifugation value is 6.0×10^9 , the nonconcentrated-procedure-without-malic-acid value will be approximately 1.5×10^8 (6.0×10^9 divided by 40). Diluting 100-fold would achieve an approximate ATP level of 1.5×10^6 . However, since the broth sample after the initial centrifugation step in the Long Centrifugation procedure (procedure 1 of Appendix B) represents a 20-fold concentration of the original urine sample, the broth sample would have to be diluted 20×100 or 2000-fold to achieve an ATP light unit level

of about 10^6 in the A_0 . By examining various long centrifugation values in this manner, cutoff points can be established that define a range of values for which the sample would need to be diluted 10-fold, 100-fold, and so on.

Clearly, the final ATP level of the A_0 reached by these manipulations can only be an approximation. ATP levels in bacteria vary from one organism to the next. In dealing with a urine of unknown infection, this factor cannot be overcome. Nevertheless, a workable range for the ATP level can still be achieved.



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